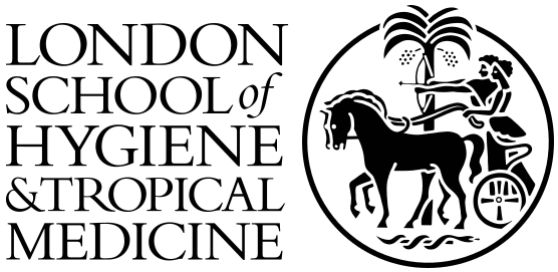
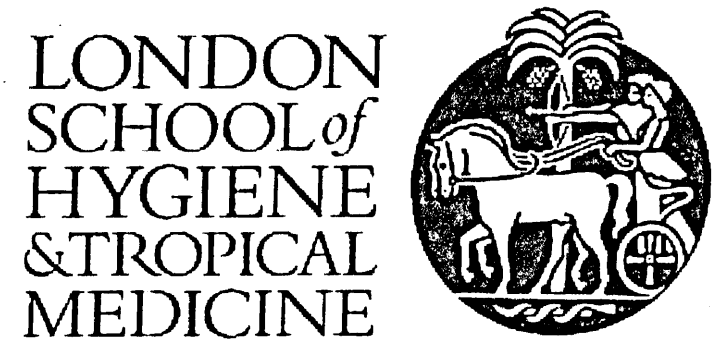


Dried Blood Spots to Improve Patient Management in Remote Settings



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Abstract

The overall aim of the thesis was to assess the feasibility and utility of using blood collected on filter paper, dried blood spots (DBS), to improve patient management in remote settings. The use of DBS was evaluated for patient monitoring, quality assurance of point of care testing, and for surveillance to improve syndromic management.

Systematic literature reviews, laboratory studies, and implementation of DBS based diagnostics were performed to answer these objectives. DBS is a feasible alternative to plasma in monitoring viral load in HIV patients on treatment. The type of filter paper, collection, storage and processing methods are critically important to obtain valid results for monitoring HIV.

A DBS protocol was evaluated for syphilis serology, giving an adjusted sensitivity and specificity of 97.7% and 99.4% compared to plasma. DBS was implemented as quality assurance for Point-of-care test (POCT), which showed that health worker proficiency varied significantly between clinics, stressing the importance of quality assurance.

Laboratory evaluations revealed the possibility of obtaining adequate sensitivities to detect febrile illnesses; malaria, dengue, leptospira, B.bacilliformis, and chikungunya, but not for R. typhi and O.tsutsugamushi by DBS. When implemented in Peru, a local B.bacilliformis outbreak was identified which proved that DBS was more sensitive than the current surveillance method.

The goal of this research was to determine whether DBS can improve patient management in remote settings. As new technologies are being developed rapidly, the use of simple and robust tools such as filter paper has continued to provide an affordable and reliable solution for clinical sample collection. While DBS is not the best sampling method, it is the simplicity and robustness that makes it useful in remote settings. This research showed that DBS can be used for monitoring HIV patient on antiretroviral therapy, the quality of syphilis diagnosis, and determining the major causes of fever in children to improve recommendations for syndromic management.

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This thesis would not have been possible without the advice and support of my supervisors Rosanna Peeling and David Mabey. It has been an honour to be Rosanna's first doctoral student at the school and I am thankful for the excellent example she has provided over the years. David Mabey's unlimited aid in writing up has been greatly appreciated. Without him, it would not have been possible to finish this PhD in three years.

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Abbreviations

A

Adjusted relative risk ratios
ARR 139
Antenatal care clinics
ANC 106
Antiretroviral Treatment
ART 22

B

Basic local alignment search tool
BLAST 151

C

Centre for Disease Control and Prevention
CDC 37
Cycle point
CP 177

D

Disability Adjusted Life Years
DALY 15

E

Early infant diagnosis
EID 22
Enzyme Immuno Assay
EIA 106
Enzyme Immunosorbent Assay
EIA 50
Enzyme-linked immunosorbent assay
ELISA 50
Epstein-Barr virus
EBV 66
External Quality Assurance
EQA 20

F

Food & Drug Administration
FDA 20

H

Healthcare workers
HCW 23
Hepatitis B virus
HBV 62
Hepatitis C virus
HCV 62
Hepatitis E virus
HEV 62
Human immunodeficiency virus
HIV 15

I

Integrated Management of Childhood Illnesses
IMCI 23

L

Laboratory Information Management System
LIMS 110
Likelihood ratio tests
LTR 139

N

National Institute for Medical Research
NIMR 110
Nucleic acid amplicifation tests
NAAT 60

O

Optical Density
OD 48, 111

P

Phosphate buffered Saline
PBS 123
Phosphate Buffered Saline
PBS 52
Point of care tests
POCT 19

Q

Quality assurance
QA 22

R

Rapid plasma reagin
RPR 108
Relative risk ratios
RR 139
Royal tropical institute
KIT 152

S

Sexually transmitted infections
STI 15

T

Treponema pallidum Haemagglutination Assay
TPHA 106
Treponema pallidum Particle Agglutination
TPPA 106

V

Voluntary counselling and testing
VCT 109

W

World Health Organisation
WHO 15

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Introduction

Chapter 1

1 Introduction

1.1 Infectious diseases

Infectious diseases are a major cause of morbidity and mortality worldwide, primarily affecting children and young adults. The burden of treatable infectious diseases is substantial and is one of the main causes of mortality in developing countries (1). Human immunodeficiency virus (HIV), malaria, respiratory and diarrhoeal infections are the main infectious diseases causing illness in developing countries (2).

The burden of infectious diseases is commonly measured in number of deaths, but the World Health Organisation (WHO) and the World Bank have suggested to measure the impact by Disability Adjusted Life Years (DALYs). This measure combines years of life lost, as well as the decreased quality of life into one figure. Infectious disease account for a staggering ~325 million DALYs per year, according to WHO (2). While HIV is a significant contributor to this number, other sexually transmitted infections (STI) such as syphilis, which is responsible for an estimated 520,000 adversely affected pregnancies and neonates per year, should not be ignored (3).

Most of these deaths are preventable if the infections are identified early and appropriately treated.Correct identification of infectious causes of illness without appropriate tools is a considerable obstacle to effective clinical management in developing countries.

1.2 PATIENT MANAGEMENT

Patient management refers to the clinical care given to patients, including diagnosis and treatment, management for the chronically ill, and other clinical services offered to the population or on individual level. Clinical diagnoses are based on examination of signs, symptoms, and laboratory tests.

> *"Diagnosis is the act or process of identifying or determining the nature and cause of a disease or injury through evaluation of patient history, examination, and review of laboratory data"*
>
> (The American Heritage® Dictionary of the English Language, Fourth Edition)

While diagnoses in developed countries rely heavily on diagnostic tests, this is not the case in many developing countries. The lack of laboratory support prevents clinicians from making accurate diagnoses and evidence based clinical decisions. For example, febrile children are presumptively treated for malaria in areas of high endemicity, although their febrile illnesses may be caused by bacteria or viruses (4).

Appropriate diagnostics could substantially improve the diagnosis and management of infectious diseases in developing countries and with appropriate prevention and therapy, reduce their burden. Diagnostic tests are used for various purposes such as patient management, epidemiological studies providing surveillance of disease prevalence, outbreak investigation, guiding of clinical decision making, drug and vaccine research, and to monitor progress towards disease elimination. During infectious disease outbreaks, accurate detection of the pathogen causing the outbreak can enhance and accelerate the response (5-7). The clinical management of malaria infected children when better laboratory tests became available is an example (8, 9). Diagnostic tests are an essential component in control and prevention strategies in both developing and developed countries. More specifically, the use of diagnostic assays has become a key aspect of country policy guidelines and national public health strategies to reduce morbidity and mortality in developing countries. Diagnostics are particularly useful in national public health strategies for the detection of sexually transmitted infections (STIs)

as most of these infections are asymptomatic. Undetected infections can result in serious complications such as infertility (10-13).

Diagnostics for patient monitoring has become increasingly valuable in treating infectious diseases, such as HIV, in which the disease needs to be monitored to ensure drug regiments adherence and identify emergence of drug resistance (14).

Diagnostics are essential for the elimination of infectious agents and vaccine development. As efforts to eliminate malaria in certain settings showed, sensitive and early detection of malaria cases are essential to prevent further spread of Plasmodium species (15). Sensitive and specific diagnostics are particularly needed for the detection of malaria infections in symptomatic and asymptomatic carriers as disease prevalence and parasite density decrease. (16).

1.3 Diagnostics in developing countries

Disparities in the quality and accessibility of healthcare for patients around the world are vast and given the opportunities diagnostics offer, healthcare can be improved globally by increasing accessibility to diagnostics. Urdea et al. estimated the potential impact that accessible diagnostics would have for various infectious diseases (17). More accessible syphilis diagnostic tests that are at least 86% sensitive and 72% specific could save ≥ 138,000 adjusted life years and avert ≥148,000 stillbirths (17). A new diagnostic test for chlamydia and gonorrhoea with a sensitivity and specificity of at least 85% and 90%, could save three million DALYs and prevent >161,000 HIV infections among female sex workers.

Although millions of DALYs potentially saved when accessibility is improved, the difficult trade-off between sensitivity and accessibility of diagnostics is also shown. While assays (with close to 100% sensitivity and specificity) are commercially available for STIs, these assays are often not suitable to be used in rural health settings (18). By accepting a loss in performance but gaining an increase in accessibility, a cost-effective and most importantly, large health impact could be obtained (19, 20).

Access to accurate diagnostics is often limited due to a variety of factors related to the clinical samples and diagnostic assays used. As most assays are developed and validated for laboratory settings in developed countries, environmental control and mechanical equipment requirements could limit their use in developing countries. For blood based screening tests, venous blood needs to be obtained by a trained phlebotomist, centrifuged and shipped cold to a laboratory. Additionally, costs, technical complexity, source of electricity, cold storage requirements of kit reagents, laboratory infrastructure requirements, and the necessity of trained and skilled laboratory technicians could be reasons why certain assays are not used or applicable in developing countries (1).

To aid the development of diagnostics suitable for developing countries, characteristics of the ideal diagnostic tests were established and summarised as "ASSURED" (1). ASSURED is an acronym for; affordable, sensitive, specific,

user-friendly, rapid, equipment free and delivered to those who need it (1). The development of diagnostic assays fulfilling the ASSURED criteria has almost been achieved for the detection of certain infectious diseases. Syphilis point of care tests (POCT), fulfils some of the ASSURED criteria, as these are affordable (less than US $1) and provide a result within 20 minutes, allowing patients to be tested and treated on the same day. As POCTs are stable at room temperature and do not require additional equipment, they are highly accessible. As the use of POCT expands rapidly across the world, POCTs for HIV and in some cases syphilis are incorporated in governmental infectious disease programmes and have become the backbone of national routine screening services in various developing countries (10). However, as testing is decentralised, ensuring the proficiency of health care workers in performing the testing becomes more problematic. DBS can potentially play an important role in quality assurance of POC testing.

1.4 Evaluation of diagnostics in developing countries

Commercially available assays that are sold in the United States (US) or Europe need to obtain approval to be marketed by regulatory bodies, such as the United States Food & Drug Administration (FDA). FDA approval can be obtained when the product completes stringent tests successfully. Besides the evaluation of the sensitivity and specificity of a laboratory assay; robustness, accuracy, stability of reagents, safety control, labelling, and package materials are assessed. Laboratories performing diagnostic tests ought to assure the quality by participating in regular External Quality Assurance (EQA) programmes.

Even though commercially available assays approved by FDA are thoroughly validated, verification and validation is still needed when these assays are implemented in any laboratory around the world (21). There are various factors influencing test performance that should be taken into account when applying diagnostics. Environmental influences, such as temperature, can profoundly affect the quality of kits, and affects sample integrity. Adequate training and, for some tests, subjectivity of reading the results can influence the reproducibility and reliability of a test (4). Additionally, co-infections, suppressed immune systems, and differences in characteristics of the population or pathogen, including genetic variation, are all factors influencing diagnostic testing (22, 23). Potentially one of the main reasons why assay performance differs from the manufacturer's claims is poor sample quality. Regardless of which test is used, poor quality samples will not provide trustworthy results. The evaluation of diagnostics in developing countries should cover the complete process of shipping kit reagents, collecting samples and performing tests in a representative setting where the test would be used. The Nature Reviews Microbiology 2006 supplement contains guidelines to aid laboratory technicians in validating diagnostic assays (24).

1.5 Potential uses of dried blood spots to improve patient management

As mentioned in section 1.1, accessible diagnostics could substantially improve the diagnosis and management of infectious diseases in developing countries. POCTs are helping to increase accessibility of diagnostic tests in developing countries, but there is still a large unmet need for improvement (4). As diagnostic assays and sample collection methods are both limiting the use of laboratory based assays in developing countries, alternatives need to be found. Since the development of new diagnostic tests takes five to ten years (25), other solution needs to be sought to improve accessibility and applicability of existing diagnostics in developing countries in the near future.

To improve patient management, an alternative sampling method to venous blood (i.e. dried blood spots) was assessed, which can be easily collected, stored and transported to centralised laboratories. The collection of whole blood on filter paper i.e. dried blood spots (DBS) has shown to be a valuable tool for the collection and transportation of whole blood in various developing countries (26). As noted in Section 1.2, diagnostics are used for a variety of aspects in infectious disease control and prevention strategies, and play a pivotal role in many patient management services. Given the variety of unmet needs for improvement in patient management, three purposes of diagnostics in patient management were selected for this thesis. These purposes are; patient monitoring for HIV viral load, quality assurance for POCT, and surveillance of causes of fever. The uses of DBS to improve accessibility and quality for each of the selected diagnostic purposes were assessed for infectious diseases that are a major public health concern. Additionally, these three distinctive public health concerns were chosen potentially to enable transferability of the work to other infectious disease disciplines and health concerns. A more detailed description of the severity of the health concerns are given in the introduction of each chapter.

1.5.1 *HIV PATIENT MONITORING AND EARLY INFANT DIAGNOSIS*

When HIV POCTs were rolled out, HIV testing became accessible in remote African locations (27). With the focus of international organisations as WHO and UNAIDS on making HIV treatment accessible for all HIV positive patients, patient monitoring need to be accessible as well (14). Methods such as CD4 counting and viral load measurements are essential for the initiation and management of the rapidly increasing number of HIV positive patients on antiretroviral therapy. Access to these tests in developing countries is a concern (28). It is not expected that CD4 counting or HIV VL assays will become available for use in remote locations within the coming few years (29). With over 8 million patients on Antiretroviral Treatment (ART), HIV VL monitoring is becoming increasingly essential to ensure compliance with drug regimens and that patients are switched to an effective treatment when resistance develops. While efforts are made to develop a POC HIV VL assay, alternative solutions are needed for now and the near future. The WHO is reviewing DBS as an option to improve access to viral load monitoring and early infant diagnosis (EID) by collecting samples remotely and performing tests at reference laboratories. In order to do this, the difficulties related to collection and transportation of plasma tubes need to be solved for use in remote settings as phlebotomists and cold storage, particularly during transportation, are not available or feasible. The use of filter paper for the collection of whole blood, DBS, has several advantages over venous blood collection. DBS can be implemented as an easy and inexpensive means of collecting and storing blood specimens under field conditions, while blood is collected by finger or heel prick (30-34). The reduction in materials required, biological waste produced and the fact that no phlebotomists are required at clinic level, considerably decreases the costs and ease of sample collection compared to venipuncture (35). DBS could potentially increase the accessibility of HIV VL and EID testing to remote areas (36-38).

1.5.2 *QUALITY OF POCT*

Due to the fact that the use of POCT in developing countries is expanding rapidly, the need for a continuous quality assurance (QA) method becomes

urgent (14). With POCT, testing performed by healthcare workers (HCWs) takes place at bed sides or at remote health clinics. Monitoring the quality by QA is, therefore, not always feasible. The potential risks of offering POCT without QA could have significant consequences. Because of the lack of QA and opportunity to retest a percentage of patients on a regular basis, the uncertainty and unawareness of the performance of POCTs are worrisome. To achieve the maximum potential of POCTs, QA systems need to be developed (4). DBS samples have been suggested as a suitable QA method for ensuring the quality of HIV POCTs, but this has not been evaluated in practice (39).

1.5.3 SYNDROMIC DIAGNOSES

WHO treatment guidelines for children are documented in the Integrated Management of Childhood Illnesses (IMCI) guidelines. The syndromic management of childhood illnesses is stated in these guidelines, to aid policy makers and healthcare practitioners in resource constrained settings. The guidelines state that, in malaria endemic regions, children should be treated for malaria when diagnosis is not available by laboratory testing or POCT. When malaria POCT was incorporated in the IMCI treatment guidelines, overtreatment of malaria became apparent in various regions of the world. Recent results indicate that malaria prevalence is decreasing while treatment is increasing (8, 40). Various "non-malarial" febrile pathogens can cause severe illness and in those cases appropriate treatment to prevent health consequences is required. The frequent overtreatment of malaria in areas where fever can be caused by dengue, rickettsia, and other febrile illnesses, leads to the unnecessary use of anti-malarials which in turn leads to a reduction in overall health benefits (41).

Distinguishing between benign and serious causes of fever in young children is a cause of concern for HCWs all over the world. The differentiation between viral infection and "occult bacteremia" (clinically significant presence of bacteria in the blood stream) is the main distinction that mostly relies on laboratory tests (42). The great range of possible causes and often the lack of laboratory tests, forces HCWs in developing countries to start treatment without clear evidence.

The bacterial or viral causes of fever in children often show epidemic, endemic and seasonal characteristics that can vary from area to area. These characteristics make diagnosis without laboratory support complex. When the incidence and prevalence of the various fever associated pathogens in children are monitored, care could be improved. Fever associated pathogens such as malaria, dengue, rickettsias, leptospira, or chikungunya would require surveillance to determine the prevalence and incidence accurately in febrile children from rural areas. Surveillance method should include locally prevalent infectious diseases. To give an example, Bartonella bacilliformis, is a life threatening febrile infection and outbreaks occur in Peru (43, 44). As the symptoms are relatively comparable to other febrile infectious diseases, such as malaria or dengue, B.bacilliformis remains misdiagnosed, and prevalence data are not available.

To improve the syndromic management of febrile children, a surveillance system would be a suitable option to empower governments and clinicians with prevalence data, to develop evidence based guidelines, and provide children with adequate treatment.

1.6 AIMS AND OBJECTIVES

Hypothesis:

DBS can improve patient management in developing countries

Objectives:

1. To assess if patient management could be improved by using DBS samples.

    a. To conduct a systematic review of the published literature on the applicability of filter paper sampling in the diagnosis of infectious diseases.

    b. To conduct a systematic review of the applicability of DBS in HIV viral load patient monitoring and early HIV infant diagnosis.

2. To assess if DBS can be used to assure the quality of point-of-care testing in developing countries.

    a. To evaluate syphilis POCT performance before implementing the quality assurance method.

    b. To evaluate DBS as an alternative sampling method for obtaining quality control samples for syphilis.

    c. To implement central laboratory testing of DBS as a quality assurance method for HIV and syphilis POCT.

3. To assess if DBS can be used for detecting causes of fever in children.

    a. To develop and implement a surveillance method based on DBS for identifying the infectious causes of fever in children, in a proof of concept study.

1.7 Study rationale

To address these objectives, the thesis requires a clear conceptual framework to harmonise and structure the research efforts of using DBS samples, potentially to improve clinical management in developing countries.

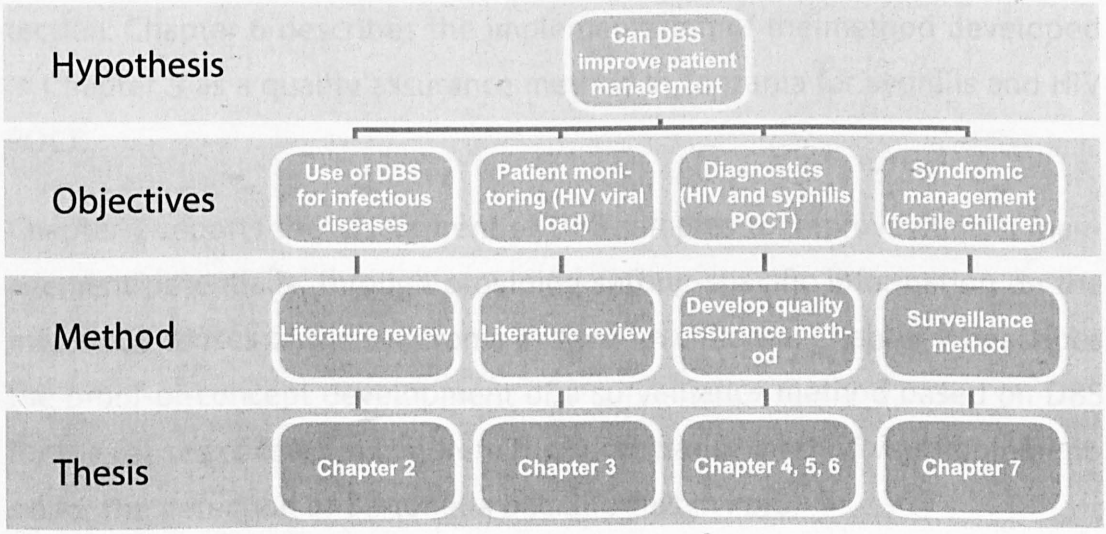
Science is facts; just as houses are made of stones, so is science made of facts; but a pile of stones is not a house and a collection of facts is not necessarily science.

Henri Poincare (1854 - 1912)

Although the use of DBS will be discussed in considerable detail, this thesis focuses on the possibility of improving clinical and laboratory based diagnoses, and providing solutions to unmet needs in developing countries.

A schematic overview of the objectives and methods is given in Figure 1.2.

Figure 1.1: Schematic overview of the PhD thesis



To illustrate the objectives in Figure 1.2, objectives 1a and 1b are presented separately while grouped as one objective in the previous section (study rationale).

1.8 Structure of thesis

The thesis consists of six chapters following the three objectives. Chapter 2 describes the literature, to identify potential uses of DBS for diagnostics in developing countries. This chapter provides a complete overview of DBS utility for infectious diseases, specifically focused on the detection of infectious diseases by using nucleic acid or serology based assays.

Chapters 3-6 comprises of four research papers, each with an introduction. In Chapter 3, the results of a systematic literature review are presented on the use of DBS for HIV viral load monitoring and HIV early infant diagnosis. Both Chapter two and three address objective 1 of the thesis.

Objective 2 is covered by three research papers in Chapters 4-6. Chapter 4 depicts the evaluation of the performance and accessibility of two syphilis screening assays in Tanzania, and the trade-off between assay performance and accessibility is discussed in detail. Chapter 5 reports on the development and validation of filter paper based Treponema pallidum antibody detection. Chapter 6 describes the implementation of the method developed in Chapter 5 as a quality assurance method in Tanzania for syphilis and HIV POCT.

Chapter 7 reports the assessment of DBS samples to improve clinical management potentially, through providing setting-specific information on the infectious causes of the syndrome of fever in children. Chapter 7 describes the proof-of-concept development of a surveillance method based on DBS for the causes of fever in children. The surveillance method was implemented for the detection of Bartonella bacilliformis in Peru.

1.9 Contribution of the candidate to the thesis

Chapter 2 contains two sections: the first section (overview of DBS methods) was independently designed and executed by the candidate, and the second (DBS systematic literature review) was undertaken by the candidate and Ivo Elliot. The systematic literature search was performed by the candidate in collaboration with Ivo Elliot and supervised by Paul Newton and the candidate's supervisors David Mabey and Rosanna Peeling. The first section will be used for the WHO technical brief on HIV VL on DBS, and the second section will be submitted to Lancet infectious diseases journal. The candidate will be first author, with an equal contribution note to Ivo Elliot as most of the work was done in duplicate, to make the literature review systematic.

Chapter 3 was based on the assignment of WHO to perform a literature review regarding the evaluation of HIV viral load methods. The literature review will lead to a peer reviewed publication and technical brief. This was delegated by Rosanna Peeling to the candidate and Kimberly Sollis. The candidate initiated an additional literature review for HIV viral load measurements on DBS samples. When WHO reviewed the additional DBS sections, a separate article and technical brief for DBS was requested. Only the DBS literature review is included in Chapter 3. The systematic literature review for HIV VL and EID for DBS samples was performed by the candidate, Kimberly Sollis proof read earlier versions of the manuscript. The literature review was supervised by Rosanna Peeling.

It is amazing what you can accomplish if
you do not care who gets the credit

Harry S Truman (1884 - 1972)

The work conducted on Chapters 4, 5 and 6 was linked to a research project funded by the Bill and Melinda Gates Foundation for the implementation of syphilis POCT in seven countries. Chapter 4 was developed by the candidate and supervised by his supervisors. The laboratory tests were executed in collaboration with laboratory technicians at National Institute for Medical Research (NIMR) and the TAZAMA team in Tanzania. The TAZAMA team (Jim

Todd, Basia Zaba, and Mark Urassa) were willing to share their samples for this project and contributed to the project proposal by obtaining ethical approval and making it possible to implement this project into their on-going study. Chapter 5 was developed by the candidate in collaboration with the candidate's supervisors, and the laboratory tests were performed in collaboration with NIMR laboratory technicians. Chapter 6 was designed in collaboration with the candidate's supervisors and performed in collaboration with Julius Mngara who maintained contact with HCW during the study period and Thomas van der Vlis, who assisted in handling the samples in the laboratory.

Chapter 7 was based on a grant obtained by the candidate in collaboration with his supervisors. The project was developed and conducted independently. Paul Newton, Dave Moore and Patricia Garcia collaborated by making it possible to use their samples or generously made it possible to use their research setting and network. David Mabey and Colin Sutherland provided feedback on the grant report, which resulted in this thesis chapter.

The use of DBS for infectious disease detection

Chapter 2

2 The use of DBS for infectious disease detection

2.1 History

The use of paper to store clinical samples for subsequent analysis has a long history. In Germany, during World War II, dysenteric faeces were dried on filter paper and Shigella subsequently identified (45). In 1950 Joe et al. in Leiden, The Netherlands received faeces dried onto filter paper by post from Indonesia and also successfully detected Shigella (46). Robert Guthrie is widely credited as being the first to use blood dried on filter paper (so-called Guthrie cards) to diagnose phenylketonuria in neonates in 1963 (47). However, in 1957 Wolff in Leiden had already noted the fruitlessness of performing serological analysis on blood sent from the tropics and was investigating the use of filter paper as a possible alternative(48). In 1963 van Thiel collected blood samples on filter paper in West New Guinea and was able to detect antibodies to leptospirosis in Leiden, even after the filter paper was stored for a year in a sealed box at room temperature (49). Since then, filter paper has become a widely used method of storing and transporting diverse specimen types, from humans, animals, and plants, for analysis. From humans, almost all types of body fluids, from blood to saliva and faeces to breast milk, have been stored on filter paper.

The collection of blood by filter paper has been applied in various research and clinical settings. Dried Blood Spots (DBS) have been applied to surveillance (33, 50-55), diagnostics (56), screening (57), drug resistance screening (58-60), therapeutic drug monitoring (61), and as a quality control method in various research and clinical settings around the world (39, 62).

When performing diagnostic or epidemiological surveys, particularly in remote areas in resource-poor settings, the facilities for processing whole blood and maintaining frozen samples are frequently non-existent. Dried blood spots (DBS) provide an ideal and inexpensive means of potentially overcoming these difficulties. Samples such as finger-prick blood are easily and quickly collected onto filter paper and shipped at room temperature, hence allowing the samples to be sent by post. The reduction in required

materials, reduced biological waste production and minimal training of personnel at clinic level, decreases costs considerably in comparison to standard blood collection by venepuncture (35). Additionally, the small size of DBS -a little bit bigger than a credit card- allows easy storage and use after prolonged periods. DBS has shown to be particularly useful in increasing uptake of testing services (63, 64).

Blood sample volumes on filter paper are inevitably small and rigorous assay validation must be performed to achieve suitable sensitivity and specificity. For certain pathogens and sample types long-term storage at ambient temperature, particularly when exposed to high humidity, will reduce sensitivity.

2.2 RATIONALE

The use of DBS in infectious disease detection has been reviewed in the context of epidemiological studies (65), HIV detection and monitoring (36, 66-70), virology (26), cytomegalovirus (71, 72) and for drug assays (73). However, there are no recent clinically-orientated reviews of DBS for the diagnosis of infectious diseases. A review on the current use of filter paper, focusing on evaluation of DBS assays compared to a recognised gold standard for the diagnosis and or epidemiological study of infectious diseases for both nucleic acid amplification and serological assays, was performed.

The aim of this literature review was to provide a complete overview of literature describing both technical details and uses of DBS samples for infectious diseases. In this review, the objectives were (1) to assess the general implications of using DBS instead of traditional sampling methods and (2) to assess the use of DBS for the diagnosis or surveillance of infectious diseases. Objective 1 is addressed in section 2.3. For objective 2, discussed in section 2.7, the use of filter paper with samples other than whole blood is briefly reviewed as well. As filter paper samples are also used for veterinary health with little overlap with human health, a brief summary of this parallel work, particularly for livestock diseases with significant economic impact, was included in section 2.7.

2.2.1 METHODS

For section 2.3, the literature review and additional information was obtained from manufacturers and websites to provide a detailed description of available techniques. This is presented as a review of DBS techniques and key considerations. Section 2.7 is solely based on a review of studies evaluating the performance of DBS for infectious disease detection. Standard guidance in performing the review as documented by the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) checklists was followed (74).

2.2.1.1 Inclusion criteria

Inclusion criteria were defined using PICOS (Population, Interventions, Comparisons, Outcomes, Study Design) criteria (75). Studies evaluating the performance of DBS for the detection of infectious diseases against a valid reference sample were considered for inclusion. Both DBS and a valid reference sample had to be performed on the same commercially available assay, when applicable DBS.

Included studies evaluated the use of DBS compared with a recognized gold standard reference sample (e.g. serum, plasma or whole blood) for qualitative and or quantitative analysis of nucleic acids or serological (antibody/antigen) testing to detect any human or animal pathogen. Where commercially available assays are routinely employed in diagnosis, in-house methods or outdated commercial assays were excluded. The following diseases had in-house assays excluded after agreement between all reviewers: human immunodeficiency virus (HIV), hepatitis B and C, cytomegalovirus (CMV), measles and rubella. Excluded articles are listed in Annex 10.2.8.

2.2.1.2 Information Sources

Studies were identified by searching the electronic databases MEDLINE and Embase. The search was performed on 13th December 2011. Additional relevant studies were identified by reviewing the references of the included studies.

2.2.1.3 Search Strategy

We used the following terms to search the electronic databases and made use related search tools when applicable:

"dried blood" , "blood spot", "blood spots", "dried serum", "serum spot", "serum spots" ,"filter paper", "filter cards" ,"filter disc", "filter discs", "filter disk", "filter disks" ,"blotting paper", "Guthrie card", "Whatman paper", "Isocode stix" ,"FTA paper", "FTA card", "filter paper", "disease" and made use of corresponding Medical Subject Heading (MeSH) terms for the above keywords.

2.2.1.4 Study selection

Titles and abstracts were screened for relevance. Full text articles of potentially eligible studies were then obtained and assessed against inclusion criteria. Reasons for exclusion were recorded. The study selection process was performed in the first instance by PWS and IE. Where uncertainty about eligibility existed, studies were discussed and eligibility was decided by consensus. The study selection process is summarised in the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) flow diagram shown in Annex 10.2.2.

2.2.1.5 Data extraction process

A data extraction form was developed and tested prior to use in the review. Information was extracted and entered into a Microsoft Excel datasheet. Quantitative data extracted included: study site, filter paper type, sample and reference sample type, paper storage conditions and details of the assay test (manufacturer or in-house reference, punch size, elution volume and conditions, extraction kit and assay input volume). Descriptive data included any relevant additional outcomes. See full review protocol in Annex 10.2.1. Two reviewers independently extracted data from the included studies. Disagreements were resolved by consensus.

During the selection process studies examining the practical aspects and implications of using DBS compared with non-filter paper samples (either in addition or separately to evaluating DBS) were identified. These articles include detailed studies, reviews and guidance on key areas including: filter paper types, DBS preparation, drying, transportation, biohazard risk, storage and stability, punch size, punch disinfection, and elution methods.

A non-exhaustive selection of studies on the use of filter paper with samples other than whole blood and for animal pathogens were identified. Where available, sensitivity and specificity of assays performed on these samples were recorded in comparison to a gold standard reference sample. Additional practical information concerning the use of these sample types was also recorded where relevant. Lastly, a limited selection of studies on animal pathogens – both zoonotic and non-zoonotic – and particularly those

important in veterinary health were identified during the initial selection process and kept separately. General descriptive information on the use of filter paper in this field was extracted.

2.3 Review of methods and key considerations

2.3.1 Types of filter paper

Filter paper compounds include cellulose, glass, quartz fibre filters, nylon, PVC, polyvinylidine and many other filter membranes. Cellulose filters derived from cotton are most commonly used in infectious disease research. There are two types of cellulose filter paper; treated and non-treated filter paper of which the non-treated is more commonly used. There exist many versions of the 100% cellulose filter paper, varying by thickness and pore size among the many manufacturers that produce comparable cards. The 100% cellulose filter paper is widely used in neonatal screening (76). For the collection of whole blood, the thickness is of considerable importance as the compactness and volume of blood on the filter paper can vary tremendously. For laboratory tests, a small section of blood containing paper is removed and used for further analysis. The type of paper will therefore influence the blood sample volume that is processed. The quality of paper e.g. the thickness and consistency of filter paper is essential to standardise and reduce variability within and between samples (76).

The treated filter paper types often contain lysis buffers, impregnated on the filter paper membrane. This method lysis the cells and reduces contamination risks. It additionally –according to the manufacturer – leads to greater stability and inactivity of enzymes that are potentially harmful for nucleic acids. Most treated cards can only be used for nucleic acids and are not suitable for serology or chemical analysis. Although many types of filter paper are used in infectious disease detection, we selected four papers, 2 treated and 2 untreated filter papers. These two treated filter papers are regularly used for infectious disease detection and the 2 untreated filter papers are the only two approved by the FDA and have CE marking (*Conformité Européenne)* for human whole blood sampling (filter paper grade 903 of Whatman and Ahlstrom grade 226 of PerkinElmer). Every lot of these filter papers are checked by US Centre for Disease Control and Prevention (CDC) to ensure the correlation between spot size and whole blood volume varies minimally (77). The table below gives details of four filter paper types used for the detection of infectious diseases (Table 2.1). Details were obtained from the manufacturers.

Table 2.1: Commonly used filter paper types, (manufacturers' details)

Paper cards	903	FTA	FTA Elute	226
Manufacturing company	Whatman part of GE Healthcare	Whatman part of GE Healthcare	Whatman part of GE Healthcare	Perkin Elmer
Material	100% cellulose	Treated cellulose	Treated cellulose	100% cellulose
Adjustable format	Yes. Custom formats are available as well as standard catalogue items. This is an untreated matrix that is suitable for antibody testing as well as nucleic acid testing.	Yes. Custom formats are available as well as standard catalogue items. This is a treated format that is suitable for nucleic acid testing.	Yes. Custom formats are available as well as standard catalogue items. This is a treated format that is suitable for nucleic acid testing.	Yes. Custom formats are available as well as standard catalogue items. This is an untreated matrix that is suitable for antigen testing as well as nucleic acid testing.
Number of spots	Standard format has 5 circles, 12- 13mm diameter. Custom formats can have as many circles as needed	A few standard formats are available, 1 – 4 circles, 1" in diameter. Custom formats can have as many circles as needed	Standard format has 4 circles, 12- 13mm. Custom formats can have as many circles as needed	Standard format has 5 circles, 12- 13mm diameter. Custom formats can have as many circles as needed
Quantity of whole blood per spot	75 – 80µl per circle	100 – 125µl per circle	70 – 80µl per circle	75 – 80µl per circle
Recommended drying time	3 hours	3 hours	3 hours	3 hours

Paper cards	903	FTA	FTA Elute	226
Stability	Stability of > 3 months for viral RNA has been documented in reference papers	Stability of > 3months for viral RNA has been documented in reference papers. DNA is stable at room temperature for more than 10 years.	DNA is stable at room temperature for more than 10 years.	Comparable performance to 903 filter paper.
Lot variability	Not documented for HIV testing	Not documented for HIV testing	Not documented for HIV testing	
Pack quantity	100 cards per pack	100 cards per pack	100 cards per pack	100 cards per pack
FDA/ CE approved transport of blood for diagnostics	Class 2 medical device and CE marked as a sample collection device for blood	For research use only	CE marked as a sample collection device for blood	Class 2 medical device and CE marked as a sample collection device for blood
Recommended storage time and conditions	For nucleic acids: Short-term: +4°C Long-term: -20°C	Room temperature storage for DNA, +4°C for RNA	Room temperature storage for DNA, +4°C for RNA	For nucleic acids: Short-term: +4°C Long-term: -20°C
Suggestions and Comments (manufacturer's)	903 has been used since the 1990's, first with antibody testing and later HIV-1 and viral load tests.	FTA offers the advantage of pathogen inactivation, safer for handling and shipping.	FTA Elute offers the advantage of pathogen inactivation, safer for handling and shipping.	The thickness of 226 filter paper is very consistent and is therefore ideal for quantitative analysis

2.3.2 *Collection of filter paper samples*

Various protocols have been written for the collection of filter paper samples by finger prick. A basic outline is given below, obtained from Mei *et al.* (76). A detailed presentation and training session has been developed by the WHO which is useful for teaching purposes (78, 79).

Table 2.2: Collection procedures for DBS samples

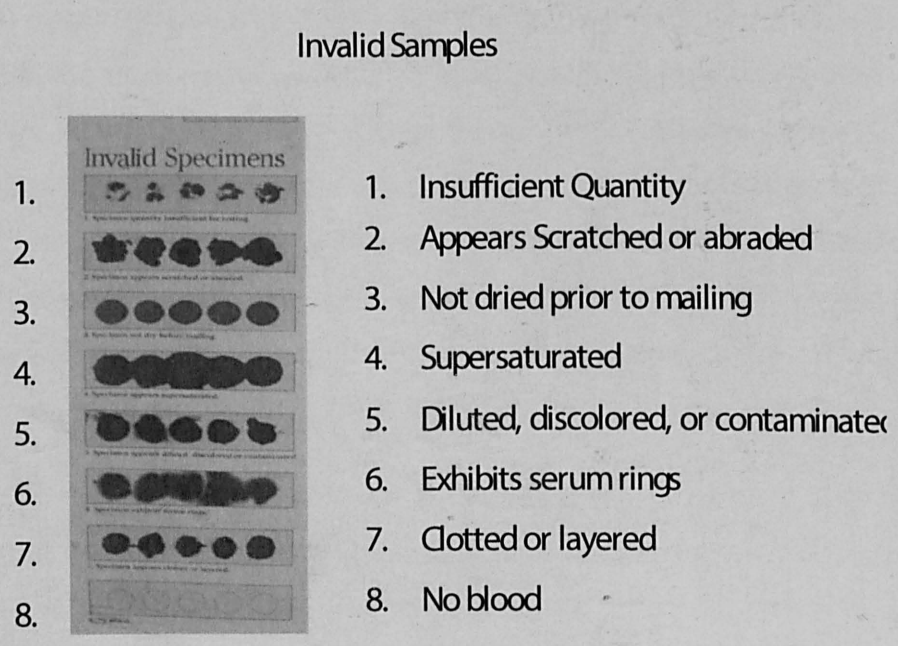
- Clearly label card with appropriate sample number or name
- Do not touch the filter paper circles before or after blood collection
- Clean the puncture site with 70% isopropanol or 70% ethanol
- Use sterile, disposable lancet
- Keep the puncture site below the heart level
- Wipe away the first drop of blood
- Apply the second drop of blood to the surface of the filter paper circle by gently pressing the paper to puncture site or use sterile micropipette
- If not completely covered, apply immediately a second drop of blood to the circle
- Fill all the circles only on one side of the filter paper
- Dry the specimen at ambient temperature for 3-4 hours in horizontal position, out of direct sunlight and protected from insects or rodents
- Avoid touching or smearing the blood spots
- Pack filter paper with desiccants and humidity indicator in a gas impermeable zip lock bag
- Insert DBS bags into rip-resistant envelope
- Include documentation and place in large envelope for transport

Although sample collection procedures are relatively straightforward, adequate training is necessary to minimize potential sources of error. Proper placement of whole blood on the filter paper is critical as the uniform absorbing properties are diminished if blood is smeared onto the filter paper. To assure blood is uniformly absorbed, a drop should be formed on the finger and contact between the finger and filter paper should be prevented. Applying multiple drops onto the same area will cause blood to layer which prevents a homogenous distribution of whole blood across the filter. More details for spotting and storing DBS samples can also be found on the website of the laboratory technologist committee (80). The importance of correct DBS preparation, storage, and manipulation conditions was highlighted by Bertagnolio *et al.*, who showed that amplification success rates for HIV drug resistance sequencing varied from 53 to 92% (66).

2.3.3 *Quality control of samples*

The quality and integrity of a clinical sample is essential for proper quality diagnostic testing, irrespective of which tests are applied. The quality of the filter paper samples upon arrival at the laboratory can vary due to environmental factors that in the end could influence test results. Effects such as humidity, contamination, mould, and overall state of the cards can be beneficial to register. Recording these effects is noteworthy because they may result in exclusion of those samples due to poor quality. Besides environmental influences that can affect the quality of DBS samples, incorrect sample collection is also a potential cause that needs to be recorded. Layered samples, small quantities of blood, serum rings, and supersaturated sampling influence the sample quality. A reference of incorrect DBS samples are given in Figure 2.1 (81).

Figure 2.1: Examples of invalid DBS samples. Obtained from NYDSOH (81).



With insufficient blood or poor sample integrity, a correct test result cannot be given. Diluted or discoloured DBS samples are indicators that the sample may not have been properly dried or became contaminated, causing the colour change. Clotted or layered cards raise the possibility that a sample contains blood of two persons spotted onto one card or that the sample was incorrectly collected. It is preferable to exclude these samples for further analysis. Serum rings can occur when alcohol used for cleaning the finger did not dry sufficiently before puncturing the finger. Serum rings can also occur due to clinical conditions such as anaemia that influence the amount of serum collected when obtaining a punch from the filter paper (see Chapter 7). In this case, serum ring samples could contain insufficient blood for valid diagnostic tests.

2.3.4 *Safety & shipment requirements*

As DBS contains dried blood, the samples should be processed as contagious material. The safety procedures and package requirements are fewer than would be the case with liquid whole blood. US CDC has shipping guidelines for DBS posted online (82). Although it is believed that bacteria and viruses have reduced activity when stored at DBS samples, Streptococci group A could be cultured after elution of DBS samples (83, 84). Standard safety measures and regulations should be in place for processing DBS samples in the laboratory. Permits may be required for international shipments but this depends on country regulations and particular circumstances. According to the manufacturer, FTA and FTA elute inactivate highly pathogenic organisms.

2.3.5 *Punching samples*

Manual or automated punch devices, such as handheld office punches or automated machines used for neonatal screening are suitable for removing a section from the filter paper (Table 2.3). Most automated punching platforms work with 96 well-plates which are ideal for serological assays such as ELISA, but are less suitable for nucleic acid detection as most extraction methods are not based on 96 well-plates. Manual punches such as office punches are an affordable option (±2 Euro), although it is necessary to attach a small tube to handheld office punches to guide the punched material to the correct well for further analysis (personal experience).

Nucleic acid extraction protocols for DBS samples in 96 well-plate format exist, both as commercially available kits and in-house methods (Qiagen 96 well-plate DNA minikit, Chelex, Roche MagnaPure röbot). Unfortunately, for most extraction methods, the eluate needs to be separated from the filter paper before extraction. Most automated extraction robots are not suited for filter paper debris as these clots the pipette tips.

A recent development is that perforated filter paper cards are now commercially available, allowing the spots to be removed with a pipette tip. These cards could remove the necessity to use punching machines, limiting the use of equipment and potential contamination risks. This currently exists for both Whatman 903 and 226 of PerkinElmer.

To assure the punches contain the correct quantities of dried blood, it is recommended to punch with the back of the card facing forward in order to visually check that the filter paper is thoroughly saturated with whole blood.

Table 2.3: Punch methods (Manufacturer's specifications)

Punch methods	Scissor	Office hole-punch	Harris-unicore and multicore	DBS Puncher (1296-071)	Multi Puncher (1296-081)	Auto Puncher (1296-091)
Manufacturer	-	-	Whatman	PartnerTech	BSD Robotics	BSD Robotics
Method overview	Manual	Manual	Manual	Semi-automated DBS punching	Semi-automated DBS punching	Automated DBS punching
Applicable DBS types	All types	All types	All types	Sheet and cassette	Sheet and cassette	Cassette
throughput	30 punches per hour	40 punches per hour	40 punches per hour	1 plate/4 minutes	NR	NR
Replacement time/ punches	NR	NR	~10 punches	400000 punches (appr.)	400000 punches (appr.)	400000 punches (appr.)
Punch sizes (diameter)	Complete circle (12mm)	3mm, 6 mm	1.5 – 8.0 mm	3.2mm, 4.7mm and 6mm	3.2mm, and 6 mm	3.2mm and 6mm
Maintenance costs	None	None	none	NR	NR	NR
Operational costs	None	None	None	NR	NR	NR
Cleaning procedures	Punch a clean card twice or use 1% Virkon with 70% ethanol	Punch a clean card twice or use 1% Virkon with 70% ethanol	Punch a clean card twice or use 1% Virkon with 70% ethanol	Piston and chute cleaning, wiping parts	Chute cleaning, wiping parts	Chute cleaning, vacuuming, wiping
Validated for PCR end-point samples	no	no	no	NR	NR	NR
Environmental requirements (temp/ humidity/ electricity etc)	none	none	none	15-35°C 10-85%RH 110-120V/220-240V 50/60Hz 60VA	10-35°C Max 80%RH @ 10-31°C -> 50%RH @ 40°C 110-240V 50/60Hz 200W	18-30°C 20-80%RH 110-240V 50/60Hz 200VA
Costs	1-3 £	1-3 £	20 £	10080 £	31164 £	68730 £

NR= not reported

2.4 Considerations when using DBS samples

There are some key considerations to be made when deciding to use DBS for the detection of infectious diseases. When implementing DBS sampling, healthcare workers need to be trained in how to collect, store and transport samples appropriately. Even though the methodology is easy, training is necessary to ensure correct sampling methods. Another key consideration is that implementing DBS in a laboratory could mean that a complete new sample flow has to be designed within that laboratory, and all necessary training and safety procedures need to be given to laboratory technicians. Besides these relatively minor limitations, the use of DBS also influences the laboratory testing procedures. When considering using DBS samples for infectious diseases detection, it is recommended to assess critically the concerns raised below.

2.4.1 Blood volume and spot size correlations

When spotting DBS with finger prick blood, it is not possible to know the exact volume of blood that is placed on the paper. As the quantity of whole blood or plasma is essential for developing and executing a reliable test, spot size is critical. A direct correlation between spot size and quantity of whole blood exists, provided the blood is absorbed on to the filter paper homogeneously (85, 86). The volume of blood absorbed correlates well with the density of the filter paper (86). The type and brand of filter paper influences the interaction between spot area and blood volume. Corran *et al.* evaluated three different types of filter paper, often used for malaria research (3MM, no. 1 and glass fibre). A two-fold difference in quantity of whole blood was found, depending on the filter paper used (86). To investigate if whole blood on filter paper spreads homogeneously, filter paper fibres were evaluated. It appeared that the volumes differed by 1-2% for each different lot of Whatman 903 and PerkinElmer 226 filter paper when compared using a central or a peripheral punch (76). This <2% difference also shows that there is minimal difference between filter paper lots (76). When comparing 903 and 226 filter papers, no more than 4-5% difference was detected for analytes that are used for neonatal screening (77). Even though blood is

homogenously spread on filter paper, applying more whole blood to each spot increases the amount of serum in a centre punch (76). Quantitative recoveries of analytes used for neonatal screening gave minimal differences by seven laboratories when using carefully created (spiked) blood samples, suggesting high consistency of recovery with DBS (77).

2.4.2 *Whole blood compounds and efects on quantity of plasma*

Most infectious disease diagnostic assays are developed for plasma or serum samples and require a large sample volume. For example, HIV viral load quantification can require 600 µL of plasma while DBS typically contain 50-100 µl of whole blood. As the quantity of DBS sample is dramatically less, it is essential to ensure that DBS contains the right amount of whole blood. The quantity of serum in whole blood that was dried on filter paper is difficult to determine but essential for protocol development. Various factors such as blood haematocrit, blood volume per spot, and technical aspects of filter paper contribute to different extraction yields of plasma in a DBS sample (87).

The relation between plasma and haematocrit levels is relatively straightforward for Ethylenediaminetetraacetic acid (EDTA) whole blood but is slightly more complicated for DBS samples. When haematocrit levels of blood spotted on filter paper increase, the amount of serum per spot (6mm) decreases (76). Additionally, because of the higher viscosity, high hematocrit blood yields a smaller DBS area than blood with a lower hematocrit. On the other hand, high haematocrit levels significantly increase levels of certain amino acids in centre punches (88). With low haematocrit levels, levels of various amino acids were higher in peripheral than in central punches (88).

If 100 µl of whole blood with a haematocrit of 55% is spotted onto filter paper (903 or 226 filter papers), a 3.2mm centre punch would contain between 1.465 – 1.487 µl of serum and a 3.2mm peripheral punch would contain 1.445 – 1.459 µl of serum (76). This estimate can be used to calculate the correct punch size to obtain the necessary serum input volume when developing a DBS protocol for diagnostic assays.

Whole blood collected by finger prick and venous blood differ from each other in composition (89). As capillary blood links arterial and venous blood flow, blood composition is different for each of these three phases. Capillary blood consists of different blood components from venous blood and when obtained by finger prick, the local trauma causes capillary blood to contain other body fluids (e.g. extra- and intracellular fluids) (89). When spotting whole blood on filter paper, the integrity of antibodies or nucleic acids could be reduced by biochemical structural changes or oxidative damage by haemoglobin. Although changes over time of the dry state of haemoglobin did not cause oxidative damage to DNA in DBS samples (90). It has been noted that storage of DNA or RNA on DBS could cause difficulties for sequencing due to tearing of DNA and RNA structures when stored on filter paper (91). In most cases, the length of DNA or RNA is adequate for general PCR and basic sequencing purposes (92).

2.4.3 *Considerations when using DBS instead of plasma/ serum*

The composition of serum and DBS eluate differs due to the presence of red blood cells and other whole blood components that would otherwise be spun down and removed. Cells such as white or red blood cells could interfere with assays and potentially reduce sensitivity. Additionally, when drying whole blood on filter paper, cell structures can be ruptured and their content released onto filter paper which could interfere with assays. These effects can be seen when higher Optical Density (OD) values are obtained for negative DBS samples that would be obtained with plasma samples. This could potentially lead to false-positives. The level of interference will vary by assay type and it may be necessary to perform additional steps to remove these interferences. For all PCR applications, this is not a problem because nucleic acids require isolation and purification from the sample.

Besides the influences mentioned above, DBS can also affect the detection of the infectious disease of interest. Depending on the pathogenic characteristics, intracellular or extracellular pathogen loads could differ between whole blood and plasma. For example, when measuring HIV viral load with DBS samples, proviral DNA as well as intracellular HIV RNA are present, which influences drug resistance and viral load measurements compared to viral load measured in plasma samples (58, 93, 94).

To give another example, dengue can be detected in plasma samples by RT-PCR. To obtain the highest sensitivity, it is necessary to obtain the samples after onset of disease, as the viral load in dengue drops after five days of fever (95). When using DBS samples, it appears that viral particles persist longer in capillary blood than in venous blood and this positively influenced the sensitivity of DBS, even when plasma or serum became negative (5, 95, 96).

As DBS spots are rehydrated in the laboratory, filter paper fibres separate from the membrane and fibres could clot filter tips when handling sample materials. It is therefore necessary to remove the DBS when using automated platforms which is currently a limitation for automation.

2.5 Considerations for using DBS samples for serological assays

Taking the previous sections into consideration, concerns, problems and potential limitations of using DBS for serological assays are included and discussed in this section in more detail. Serological antibody or antigen detection by Enzyme Immunosorbent Assay (EIA), Enzyme-linked immunosorbent assay (ELISA), or agglutination tests are the main focus of this section. These assays are widely available and commonly used for infectious disease diagnostics. In this chapter, the necessary punch size, elution buffer choices and input calculations will be presented.

2.5.1 Punch size calculation

In order to obtain a comparable sensitivity to plasma samples, ideally, the sample input volume of DBS should be equal to serum or plasma(97). It is therefore highly unlikely that a serological assay that requires 50 or 100 µl of serum would be applicable for DBS samples. Many assays dilute plasma samples to about 1:200, and these assays should be more successful candidates for DBS samples.

To assure correct DBS sample volumes are used for the assay, calculations can quickly determine the appropriate punch size and elution volume. All calculations below are based on the use of grade 903 of Whatman and filter paper 226 of Perkin Elmer. The standard printed filter paper cards of Whatman and PerkinElmer contains 5 spots, each of 12mm diameter circles. The calculations below are based on a fully covered spot with whole blood.

A punch of 3.2 mm would give 3.42l of whole blood (76). The quantity of red blood cells varies per person (haematocrit level) and this influences the amount of plasma in whole blood. To develop a protocol for DBS samples, a standard haematocrit level of 55% could be used, but this should be adjusted to match the population. When haematocrit levels are 55%, a 3.2mm punch would contain 1.47µl of serum. To translate this to a more commonly used punch size in infectious disease research, a centre punch of 6mm would roughly give 5.2 -5.7µl of serum, based on 80µl spotted whole blood sample.

2.5.2 *Punch and contamination risks*

Implementation of DBS specimens in the laboratory requires efforts to prevent and identify risks of contamination. The process of punching DBS samples could lead to contamination.

Contamination risk for serological assays is less of a concern than for nucleic acid testing. When reviewing the procedures at the national neonatal screening laboratory in the Netherlands, the Rijks Instituut voor Volksgezondheid en Milieu (RIVM), punching devices (DBS punchers) were cleaned weekly (personal communication). To exclude contamination, cleaning with bleach and alcohol is sufficient to prevent cross contamination of antibodies. Regarding the automated punching machines, fluid based cleaning protocols are complicated by the limited access to the punch head for cleaning. Cleaning the punch device by punching through a clean DBS card twice appears to be the fastest and easiest way to prevent cross- contamination (98), although it would be recommendable to perform more stringent cleaning procedures. Based on the current methods available, the automated punch devices would be a more appropriate option for serological assays as this vastly increases speed and potentially limits human errors.

2.5.3 *Sample elution*

A DBS spot can contain 5.2 – 5.7µl of serum but, as it is in a dry state, it needs to be eluted before the serum can be tested. In determining the elution volume, the punch size and elution volume is directly correlated with sample input volume and concentration necessary for the diagnostic assay. It is essential to calculate the correct punch size, elution volume, sample concentration and sample input volume for valid serological tests. This calculation can be done by using the manufacturer's protocol. For example, if 10µl of serum is diluted in 1: 100 sample diluent according to the manufacturer's protocol, a 6mm punch could be diluted in 100µl of elution buffer to obtain a 1:20 "plasma" concentration. By adding the DBS eluate in a 1:5 concentration with the sample diluent from the kit, it is possible to obtain a roughly comparable end concentration for plasma and DBS samples.

Once the sample elution volume has been calculated, determining the components of the sample elution is a vital step. The elution buffer is vital for efficient antibody or antigen extraction and reducing non-specific interference that results in background noise. The elution of antibody or antigen is done by using Phosphate Buffered Saline (PBS) or by specifically developed elution buffers (99-101). The addition of antimicrobial buffers and soap components such as Tween, , sodium azide, , merthiolate, in fetal calf serum or bovine serum albumin can be useful to minimise background noise, increase antibody recovery, and prevent microbial growth (101, 102). Different buffers and extraction protocols have been evaluated and most protocols showed good extraction performances with little differences between protocols (102, 103). The elution of antibodies from a punch can be >95% for IgG, depending on the storage length and temperature conditions prior to elution (86).

Based on the literature, an overnight elution at 4°C with PBS with 7.4pH and 0.05% Tween20 is most regularly used for serological assays such as ELISA, EIA and agglutination assays. In case of problematic elution efficiency or background noise, it would be worthwhile to experiment with different elution buffers. Knuchel *et al.* investigated the use of a quencher, which reduced background noise dramatically (104, 105).

Besides alterations to the elution procedure to suit the requirements of the serological assay, the assay procedures can also be adjusted. To improve results, it may be useful to use a stronger wash buffer and shorter or longer incubation periods if the OD value is incorrect. In some cases, the cut-off is altered to match the OD values of DBS samples (106).

When eluting a DBS spot, the spot should be white after the elution period, indicating efficient elution. If the spot is red after elution, it may have inappropriately released antibodies and antigens and the results are therefore not trustworthy (86). To validate DBS samples, OD values of matching plasma and DBS samples can be compared to estimate the recovery of antibodies (86).

2.5.4 *Storage conditions of DBS samples*

Correct storage of DBS samples is essential for valid test results. A DBS sample contains dried blood and the general consensus is to prevent humidity by using desiccants and ziplock bags. Humidity could lead to antibody and nucleic acid reduction due to protein activity (DNAse or RNAse proteins) or microbial contamination. Although the effects of humidity on nucleic acids and antibodies have not yet been proven in great detail, humidity could lead to growth of fungus. When a sample is contaminated with microbial growth, the sample cannot be used and should be removed to prevent further contamination of other samples (107). Patton *et al.* found that humidity was associated with a loss in sensitivity compared to samples stored with desiccants (23). It is recommendable to store samples dry by using desiccants and a re-sealable bag.

Various studies have examined the time DBS sample could be stored. Mei *et al.* found that *Toxoplasma* IgM recovery of samples stored for two years at -20°C was 100% compared to DBS samples at baseline (85). Although only one sample was used, HIV antibodies could be detected on a 14 year old DBS sample stored at room temperature (108).

Acceptable storage temperatures of DBS samples depend on the analyte of interest as antigen or antibody could have different storage requirements. Although it is preferable to freeze samples promptly after collection to minimize sample degradation, DBS allow flexibility for collection in field settings. Based on the literature and in the context of sample collection in remote settings, the rule of thumb would be for short term (<4 weeks) storage, room temperature (<30°C) would be acceptable, while storage for long term (>4 weeks) samples should be stored at 4°C but preferably at -20°C. Circumstances such as excessive heat and sunlight (UV) should be prevented at all times to prevent degradation of DNA and other whole blood components (76). Although DBS have been stored for over many years in unfavourable conditions and showed satisfactory results (108), it is highly recommended to follow these guidelines.

2.6 Considerations of using DBS samples for nucleic acid assays

2.6.1 *Punch size calculation*

A benefit of using DBS samples for nucleic acid detection is that the precise punch size is not necessarily relevant, unless it is for a quantitative assay such as HIV viral load. While for serological assays punch size and elution volumes are essential to be correctly related to the necessary plasma concentrations, this is not necessary for most nucleic acid amplification assays. Instead, most efforts are made to obtain the highest quantity of DNA or RNA from a DBS sample. For serological assays, most punch sizes are 3.2 or 6mm diameter while for nucleic acid assays, 6 or 12mm are more commonly used. Punch size and the associated quantity of blood processed contribute to the sensitivity for low copy numbers of DNA or RNA detection (109).

As PCR assays are becoming more sensitive, smaller sample quantities can be used. Because of this, DBS are a suitable sample type. The quantity of pathogen DNA or RNA should be high enough to be detectable by PCR when the small quantity of blood is eluted from the filter paper. Pathogens such as malaria or HIV that can reach up to a million copies of DNA or RNA per mL of blood are suitable targets for DBS, while pathogens such as *Salmonella enteric* serovar Typhi with 1 - 10 copies per mL of blood, are not.

2.6.2 *Punch and contamination risks*

When implementing DBS analysis in the laboratory, risks of contamination should be identified and prevented. The process of DBS handling could harbour contamination risks when punching samples. By automated processing of punching and extracting nucleic acids from DBS, many of these contamination risks could potentially be eliminated. A study investigated potential contamination risks of using manual handheld punchers (with three cleaning methods) and an automated punching method (Wallac autopuncher) (110). Both manual and automated methods showed little to no contamination risk when analysing HIV DNA by PCR (111, 112). In fact, the manual method with no cleaning procedure at all showed no false positive results. The automated punching machine in this study made use of lasers to

cut the filter paper, and thus is theoretically contamination free. Surprisingly, the automated punching device caused minor false positive HIV DNA results due to cross-contamination (110). Although contamination risks seem low, potential cross-contamination is still possible and precautions need to be made. Because automated punching platforms are difficult to clean with liquids, it is advisable to use hand punches which can be cleaned easily by bleach or nucleic acid degrading liquids such as DNaway. One study found that viral DNA could be transferred between samples by a punch, at least 13 times after a positive sample (113). The best method to clean a single hole punch was to use bleach, rinse in water and displace water by 100% ethanol (113). DBS samples are commonly stored in individual zip lock bags with desiccants to prevent cross-contamination, when used for nucleic acid testing. Contamination can occur more rapidly with nucleic acids then with serology as the methodologies are more sensitive.

2.6.3 *Elution & Extraction*

Various studies have reviewed the use of in-house developed protocols or adapted versions of commercially available extraction kits for nucleic acid isolation for DBS samples (109, 114, 115). Over the last few years, various manufacturers of extraction kits have developed alternative protocols to extract DNA or RNA from DBS with existing extraction methods and platforms (i.e. BioMerieux, Abbott, Roche, Qiagen). All methods are based on the elution of DBS by lysis buffer and the eluate is processed by an extraction method that resembles the standard procedures for whole blood or plasma.

The extraction efficiency of DNA from Whatman 903 filter paper has been evaluated. De Vries *et al.* compared eight different extraction methods and showed that sensitivity for the detection of cytomegalovirus (CMV) detection varies considerably depending on the extraction method used (114).

The extraction methods developed by Barbi *et al.*, Qiaamp DNA investigator kit (Qiagen, Hilden, Germany), BioRobot Universal system (Qiagen, Hilden, Germany) and MagNA Pure LC (Roche Basel, Switzerland) were the most sensitive methods. Another study evaluated the most sensitive method for CMV DNA detection, of which the phenol-chloroform extraction and QIAamp blood minikit had the highest sensitivity in detecting 200 copies CMV DNA/ mL DBS samples (109). Monleau *et al.* compared NucliSens extraction platform with Abbott preparation system for Whatman 903 filter paper DBS samples. The NucliSens extraction method provided HIV viral load results comparable to plasma samples, while Abbott platform gave minor but significant lower HIV viral loads (116). A study evaluating extraction methods with up to 27 year-old archived samples obtained the best performance with QIAamp DNA kit (BioMerieux) and EZNA Forensic DNA reagent set (Omega Bio-Tek, Norcross, USA) for Real-Time PCR analysis (115).

The extraction of DNA or RNA from FTA Elute and FTA is different to that from 903 filter paper, due to filter paper characteristics. FTA Elute has an extraction protocol that only makes use of water by washing the lyses buffer away, and DNA is eluted from the filter paper by heating at 95°C and washing out by vortexing the sample 60 times. The FTA is in that respect the opposite, as DNA remains bound to the filter paper and a small punch of 1mm can be inserted into the PCR reaction. FTA and FTA Elute provide adequate DNA yields (117, 118). FTA and FTA Elute cannot successfully be used for RNA targets (personal communication with Whatman, GE).

2.6.4 *Storage conditions of DBS samples*

When reviewing storage condition of DBS for nucleic acid testing, there are mixed messages from the literature. For example, genomic DNA could be amplified and sequenced successfully after storage of DBS at 28°C with 73% humidity for 10 years (119) while others found a 10-fold reduction of HCV RNA after 4 weeks at room temperature (120). Solomone *et al.* found HCV RNA positivity was preserved after 11 months of storage (121). For sequencing, long fragments are needed and success of sequencing seems to rely heavily on adequate storage and extraction methods (66). Mitchell *et al.* found that DBS storage at 37°C and high humidity diminished HIV-1 DNA load, compared to storage at -20°C (122). Additionally, HIV RNA seems more stable than HCV RNA, as sensitivity dropped for HCV when not stored at -20°C while for HIV it remained at comparable levels (123-125). Although Fiscus *et al.* found a 5% HIV RNA copy number loss per day when stored at room temperature for 28 days (126). The storage of FTA filter paper at room temperature containing HIV DNA was detected at similar rates as the initial test, four years ago (127).

To summarise, the different findings of storage requirements are caused by technical and biological differences which complicates interpretation of results between studies. Differences in sensitivity after storage considerably varies due to technical differences in filter paper, humidity control, drying and spotting methods, and the extraction and detection methods applied. Biological differences also influence storage effects due to the viral or bacterial load of the samples, DNA or RNA, and pathogen type (virus, gram negative / positive bacteria). Standard protocols for long term storage would be helpful for the many assays and infectious diseases, but the available data at the moment is insufficient. As the literature alone does not provide one clear recommended general storage protocol for DBS that is suitable for developing countries, personal experience was used to develop the transport and storage protocol, given below.

To provide a general guideline for storage recommendations, the recommendations below are based on storage for RNA and DNA samples stored on Whatman 903 or Perkin Elmer 226 filter papers, stored with desiccants and

adequately dried (>4 hours) before being stored or transported. For nucleic acid testing, -20°C to -80°C is appropriate for long-term storage. Sample collection, temporary storage and shipment to the laboratory can usually be done at room temperature. Although it is preferable to freeze samples promptly after collection to minimize sample degradation, DBS allow flexibility for collection in field settings. Based on the literature assessed and other guidelines reviewed online, samples collected in the field can be stored at room temperature (<30°C) and should be sent to the laboratory within 4 weeks. At the laboratory, samples should be stored ideally at -20°C when processed within 4 months. When samples have to be stored for longer, it is recommendable to store samples at -80°C when RNA, sequencing or related tests needs to be performed. Circumstances such as excessive heat and sunlight (UV) should be prevented at all times to prevent degradation of nucleic acids and other whole blood compounds. WHO Guidelines for HIV drug resistance testing are available that closely relate to the one suggested in this thesis. It is highly recommended to follow the recommendations made in this thesis or the guidelines of the WHO HIVDR for DBS storage (78, 79, 128). When samples have been stored at -20°C, it is suggested to ship samples on dry ice to prevent unnecessary thawing and freezing cycles. When removing samples from the freezer, thorough equilibration to room temperature is needed, as well as a replacement of desiccants with new ones (66).

2.6.5 *Recommendations*

Table 2.4 contains, based on the literature review and personal experience, recommendations that can be used as a starting point for serological assays and NAAT assay testing. These recommendations are a general consensus on results obtained by the literature and should be adapted to specific assay requirements, pathogens or settings where it will be used. Regarding the storage recommendations, these are developed to allow some flexibility in sample collection in remote settings. Ideally, samples are stored at -20°C or even at -80°C as soon as possible to prevent any loss of material but these recommendations provide, for most infectious diseases, an acceptable trade-off between loss of material and improved utility.

Table 2.4: Recommendations for DBS testing with serological or NAAT tests

Recommendations	Serology	NAAT
Type of filter paper	FDA approved filter paper: Whatman 903 or PerkinElmer 226	FDA approved filter paper: Whatman 903 or PerkinElmer 226
Collection method	CDC guideline for serological tests on DBS, available online (129)	WHO guidelines for HIV drug resistance testing are the best to use, available online (128)
Storage (time till testing takes place)	<4 weeks – Room Temperature <4 months – +4°C >4 months – -20°C or -80°C Humidity controlled by desiccants	<4 weeks – Room Temperature <4 months – -20°C >4 months – -80°C Humidity controlled by desiccants
Punching & cleaning	Automated puncher Cleaning: punch through clean card 1x after each sample or thoroughly clean after each plate	Manual punch Cleaning: bleach, rinse in water, displace by 100% ethanol after each punch
Elution & extraction	PBS with 0.05% tween20 eluted overnight at +4°C, potentially elute with bovine calf serum if result requires improvement	Elute with lyses buffer, at least 1 hour, preferably extracted with automated platforms as BioMerieux Nuclisens.

2.7 Review of the use of filter paper for infectious diseases

2.7.1 Results

Of the 4,011 articles retrieved (Annex 10.2.2), 3,524 were irrelevant to the study question, 92 did not evaluate filter paper and 98 were conference or meeting summaries and were excluded. Of 296 articles identified for further assessment the following were excluded - 60 that did not evaluate DBS, 7 that used tests no longer commercially available, 34 using in-house assays, 15 that did not use diagnostic assays, 31 were no suitable 'gold standard' comparator was used, 16 in non-peer-reviewed journals and 26 using non-human pathogens or non-whole blood samples. Detailed assessment of the DBS techniques of the resulting 104 articles was performed. The same 296 papers were also assessed for review of the practical aspects of filter paper use, for non-whole blood samples and for veterinary health; 192 articles were examined for this purpose after 65 were excluded as irrelevant to the study question, 23 that had no suitable 'gold standard' reference and 16 that were not peer-reviewed.

2.7.1.1 HIV 1 & 2 and HTLV 1

Efforts to make HIV testing more accessible in rural areas in developing countries, where >90% of new HIV infections occur, is critical for controlling the disease. DBS have the potential to provide a simple, robust and affordable option for collection of whole blood, screening, quality control of point of care tests, HIV viral load measurements and drug resistance testing in environments where traditional venous blood collection/transport cannot be performed (66, 130). Twenty-four studies examined the use of DBS, for the detection of HIV, compared with serum or plasma; twelve evaluated serological assays and 12 nucleic acid amplification tests (NAATs) (Annex 10.2.3).

Table 2.5: Summary of studies evaluating serological and NAAT diagnosis of HIV, comparing DBS with whole blood (DNA) and serum/plasma (RNA).

Assay type	HIV-1 detection	No. of studies	Sensitivity %	Specificity %	References
Serology	Ab / Ag	7	100	98.7-100	(131-137)
Serology	Ag (p24)	5	84-98.8	98-100	(138-142)
Serology	Western blot	1	92	100	(137)
NAAT	DNA	6	97-100	99.6 -100	(130, 143-147)
NAAT	RNA	6	99.2-100	95.6-100	(143, 148-152)
NAAT	DNA & RNA	3	99.7-100	100	(144, 147, 153)

Serological assays using DBS samples were evaluated in 13 diverse countries, thereby probably representing all HIV-1 subtypes using third generation enzyme-linked immunosorbent assays (ELISA) that detect antibodies, 4th generation ELISA tests that detect antibodies and antigens, and specific antigen tests (p24). The p24 antigen tests are used as an alternative to NAATs to detect infection in infants. Only one study examined detection of HIV-2 using DBS against sera, reporting sensitivity and specificity of 87.5% and 100%, respectively (131).

DBS have been evaluated for the detection of HIV-1 with diverse NAATs in 11 countries. Although HIV is an RNA virus, proviral HIV-1 DNA detection is commonly used for infant diagnosis. Six studies evaluated the Roche Amplicor and Roche Cobas Taqman assays on DBS samples, giving sensitivities and specificities between 97-100% and 99.6- 100%, respectively. DBS HIV-1 RNA and p24 antigen assays are increasingly used (154).

Most HIV viral load assays use quantitative Reverse Transcriptase PCR that requires large quantities of plasma (100-600µl) to transcribe RNA into DNA before amplification. Besides extracellular HIV-1 RNA amplified from plasma samples, DBS contain whole blood and therefore intracellular HIV-1 RNA and HIV-1 proviral DNA. As a result, when HIV-1 viral load assays are used with DBS, both HIV-1 RNA and HIV-1DNA will be amplified, making it potentially more sensitive than HIV-1 DNA assays. This has potential implications for early detection of HIV but also for potential overestimation of viral load.

Three studies evaluated the Roche and Abbott NAATs to detect HIV-1 RNA and DNA in DBS against whole blood. The BioMerieux HIV-1 RNA assays

cannot amplify HIV-1 DNA. False positive results by quantitative NAATs is a concern when used for qualitative purposes, but these assays remain a promising alternative for infant diagnosis (130, 150, 155). Indeed, the WHO recommends testing infants for HIV DNA, HIV RNA, or the ultrasensitive p24 antigen on plasma or on DBS samples, given that the sensitivity and specificity are >98% (154).

Two papers examined the possibility of detecting HTLV-1 serologically or by in-house NAATs, but with relatively small sample sizes. Both showed good performance compared to plasma samples (156, 157).

2.7.1.2 HEPATITIS VIRUSES

Seven studies evaluated the use of DBS for the diagnosis of hepatitis viruses (Annex 10.2.4). Three studies evaluated DBS Hepatitis C (HCV) serology against serum and plasma, finding high sensitivity and specificity >98% (103, 158, 159). Only two studies have examined the use of DBS samples for Hepatitis B (HBV) serology, yielding different performances for the three serological HBV assay types (160, 161). The potential to include HCV and HBV in HIV serology-based epidemiological studies makes testing for multiple viruses from one sample possible, and a cost-effective way to expand surveillance services. For most serological assays, a cut-off determines the positive or negative outcome of a test and can be adjusted if aimed for screening, confirmation or for surveillance. Adjusting the cut-off specifically for DBS samples could improve sensitivity and/or specificity, depending on the required balance between them (162).

The detection of HCV and Hepatitis E virus (HEV) by NAATs appear promising but more evaluations are needed before conclusions can be drawn. Tuaillon *et al.* found that HCV RNA is susceptible to degradation when samples are stored at ambient temperatures (+20°C), while Solmone *et al.* found that 100% positivity was maintained when samples were stored at 'room' temperature over a period of 11 months (159, 163). More evaluation of the optimal storage DBS conditions for HCV NAAT is required. Annex 10.2.4 summarizes all included studies.

2.7.1.3 FLAVIVIRUSES

The WHO estimates that dengue affects >50 million people each year, often in explosive outbreaks. Capture or sandwich ELISAs are used to serologically diagnose acute dengue (IgM, IgG antibodies and NS1 antigen) and estimate seroprevalence. Four studies comparing dengue ELISA using DBS and serum reported high sensitivities (>86%) and specificities (>89%) (164-167) (Table 2.6). One study reported poor correlation of DBS with serum results (166), but results were not analysed with the appropriate statistical technique (168). Antibody titres determined from DBS were more variable and lower than those from sera, suggesting a limited role in the diagnostic confirmation of acute dengue. All studies concluded that DBS IgG determination could be used successfully for seroprevalence studies. Storage conditions of one month at 'room' temperature, appeared to have no detrimental impact on results (167).

Dengue nucleic acid detection from DBS was also highly sensitive (>90.7%) in comparison to serum. The 100% specificity reported by Prado *et al.* may reflect the nature of the samples, which were prepared by spiking whole blood with dengue virus (165, 169). Consistent with the period of highest viraemia, sensitivity was highest on day one after onset of fever falling rapidly by day four. Matheus *et al.* found that dengue RNA could still be detected in dried capillary blood samples from a small number of patients 12 days following infection, whereas corresponding venous samples were negative (165). Indeed, capillary viraemia may be more prolonged than venous (170), suggesting that it would be important, in evaluation of dengue NS1 assays and NAATs, that both DBS and blood samples are compared using capillary blood. Dengue RNA was found to be stable at 37°C for a year (169). It is important to note that the virus remains viable and confers an infective risk on untreated paper during the first 48 hours after spotting on to untreated paper (169).

In a seroprevalence study of chikungunya virus, IgG was successfully detected on filter paper with 97.9% sensitivity in comparison to serum (171). Though IgM was not fully evaluated on DBS, it appeared to give similar results to those from sera (171).

Table 2.6: Summary of studies evaluating DBS for Flavivirus diagnosis

Disease, assay type & country	Author	Number of samples & filter paper type	Test	Sensitivity %	Specificity %	Notes
Dengue serology Nicaragua(164)	Balmaseda et al. (2008)(164)	169 samples Whatman No. 3	In house ELISA IgM, IgG & IgA(172)	96 IgM 93 IgA 86 IgG	89 IgM 89 IgA 92 IgG	Detecting IgM or IgA is useful for acute dengue diagnosis. IgG is optimal for dengue incidence surveillance. Danger of cross-reactivity of IgG with other Flaviviruses(164)
French Guiana	Matheus et al. (2007)(165)	130 samples Whatman paper	In house ELISA IgM(173)	89	94	
Cuba	Herrera et al. (2006)(167)	189 samples Whatman 2992	In house ultramicro ELISA(174)	92.1	98.6	IgM stable at room temperature for 1 month and at 4°C for >2 months.
Vietnam	Tran et al. (2006) (166)	781 samples Whatman 903	Dengue Fever IgM & IgG ELISA (Focus Diagnostics, USA)	NR	NR	DBS correlated poorly with serum, particularly for acute dengue infection. However, correlation inappropriate analysis(168). Limited role in diagnostic confirmation of dengue cases. IgG useful for seroprevalence studies. No effect of 1 month storage on results.

Disease, assay type & country	Author	Number of samples & filter paper type	Test	Sensitivity %	Specificity %	Notes
Dengue NAAT French Guiana(165)	Matheus et al. (2007)(165)	130 samples Whatman paper	In house PCR(175)	90.7	82.9	Serotyping also performed. Sensitivity and specificity highest, during the 1st 4 days of infection, falling rapidly thereafter.
Cuba	Prado et al. (2005)(169)	52 samples Nobuto paper	In house PCR(175)	93	100	Samples prepared with blood spiked with dengue virus. Lower limit of detection for dengue 2 than 3. RNA stable at 37°C for 1 year. Risk of viral infectivity from paper for 48hrs at room temperature.
Chikungunya Serology La Reunion	Grivard et al. (2007)(171)	144 samples Whatman 903	IgG ELISA National Arbovirus Reference Laboratory, Lyon, France	97.9	100	Seroprevalence study. IgM also detected with similar optical density thresholds as sera, but no independent quality control performed.
Japanese B encephalitis Serology Thailand	Burke et al. (1985)(176)	243 samples Nobuto paper	In house ELISA In house Haem-agglutination inhibition assay	72 38 during epidemic	NR	ELISA and haemagglutination inhibition tests were compared with serum. ELISA was more sensitive during epidemic periods. Newer, commercially available assays are available but have so far not been evaluated on DBS.

2.7.1.4 OTHER VIRUSES

Three studies evaluated measles antibody (IgM or IgG) detection using DBS samples. Uzicanin *et al.* demonstrated that the sensitivity of DBS samples, in comparison to serum, increased for IgM from 95.7% for samples collected from day 1-6, to 100% when samples were collected one week after the appearance of the rash(177). This illustrates for serological assays, the importance of the time of sample collection after the onset of the disease to obtain adequate sensitivities and that this varies between pathogens. For measles antibody tests, DBS can be stored for up to 6 months at 4˚C. However, beyond 6 months sensitivities fell, suggesting that long-term storage at 4˚C affects measles antibody stability(178).

We found only one study evaluating the use of DBS for Epstein-Barr virus (EBV) serology. Interestingly, this study compared venous and capillary blood spotted on two different filter paper types (Whatman 903 and No.3) for ELISA (EBNA1 plus VCA-p18) and found a higher sensitivity with 903 paper (179). For the detection of CMV, a serological assay and a NAAT test were evaluated between plasma and DBS. The NAAT was 100% sensitive and specific while the serological assay had lower sensitivity and specificity, but both were >93% (106, 180). Annex 10.2.5 summarizes all included studies.

2.7.1.5 MALARIA

For the detection and speciation of malaria, no commercially available DBS assays evalautions have been published in peer-reviewed journals. Two studies compared PCR on DBS against whole blood and found a lower sensitivity, particularly for samples with low parasitaemia (181, 182) (Table 2.7). DBS PCR compared to microscopy, achieves comparable performance or, in some studies, is more sensitive (9). It appears that DBS is more specific than whole blood samples when used for PCR analysis using the less sensitive microscopy as the reference method (182, 183).

The nested PCR assay developed by Snounou *et al.* in 1993(184) is commonly used as the reference method for malaria PCR. Based on the eight papers included in this review, malaria detection by PCR on DBS appears to be a suitable alternative to microscopy and are frequently used in surveillance studies(185). DBS are also commonly used for detection of malaria resistance molecular markers (186).

Table 2.7: Summary of studies evaluating DBS for malaria

Country	Author	Sample size & filter paper	assay	Pf	Po	Pv	Pm	Un-known	Sensitivity %	Specificity %	Reference test	Notes
Thailand	Long *et al.* (1995)(187)	56 samples Whatman 903	In-house (188)					✓	94.6	NR	Thin/thick blood smear	
Malaysia	Singh et al. (1996)(189)	166 samples Whatman 3MM	In-house (184) (adjusted)	✓		✓			97.4	NR	Thick blood smear	
Malaysia	Singh et al. (1999)(190)	129 samples Whatman 3MM	In-house	✓	✓	✓	✓		NR	NR		Limit of detection: 6 parasites per µl
Singapore	Tham et al. (1999)(191)	52 samples Whatman No.1	In-house (192)	✓		✓			100	100	Thin/thick blood smear	Limit of detection: 4 parasites per µl
Malaysia, Myanmar, Thailand	Mangold et al. (2005) (193)	81 samples Isocode cards	In-house	✓	✓*	✓			94.1 (Pf) 100 (Pv)	100 (Pf) 99.1 (Pv)	Thin/thick blood smear	*1 out of 1 Po samples detected
Thailand	Boonma et al. (2007)(183)	136 samples Whatman 3MM	Multiplex PCR(194)	✓		✓			100 (Pf) 92.7 (Pv)	100 (Pf) 100 (Pv)	Consensus of 3 PCR assays	Specificity of all 3 assays lower (93.8-97%) when compared to microscopy.
			Nested PCR(195)	✓		✓			100 (Pf) 100 (Pv)	99 (Pf) 100(Pv)		
			RT-PCR(196)	✓		✓			100 (pf) 100 (pv)	100 (pf) 100 (pv)		

Country	Author	Sample size & filter paper	assay	Pf	Po	Pv	Pm	Un-known	Sensitivity %	Specificity %	Reference test	Notes
Saudi Arabia	Al-Harthi et al. (2008) (181)	118 samples Whatman	In-house (197)					✓	73	NR	Thin/thick blood smear	Several microscopy negative samples positive on DBS PCR.
Thailand Zimbabwe	Yamamura et al. (2009) (198)	156 samples FTA card	In-house	✓					97.8	100	Thin/Thick blood smear	Limit of detection: 10 copies/ reaction
Iran	Ataei et al. (2011)(182)	75 samples DNA Banking Card	In-house (199)	✓		✓			97	100	Thin/thick blood smear	Whole blood more sensitive but less specific than DBS compared to microscopy (100% sensitivity, 95.2% specificity)
Kenya	Wangai et al. (2011)(9)	356 samples Whatman 3MM	In-house	✓					100	79	Thin/Thick blood smear	Low specificity potentially caused by insufficient microscopy expertise

NR= Not reporting Pf= Plasmodium falciparum, Pv= P. vivax, Po= P. ovale, Pm= P. malaria

2.7.1.6 NON-MALARIAL PARASITES

Parasites account for many neglected tropical diseases afflicting hundreds of millions of people worldwide, predominantly in resource-poor regions with limited access to diagnostic facilities are affected by parasites (200). The use of filter paper to aid with diagnosis and understanding the epidemiology of these diseases is thus very attractive. The mapping of lymphatic filariasis and monitoring of elimination programs provide an ideal role for DBS. Three recent studies evaluated serological tests for the Og4C3 antigen to *W. bancrofti* on DBS, compared to serum, giving sensitivities of >93% and specificities of 82-100% (201-203) (Table 2.8). The earliest study performed, by Gyapong *et al.* in Ghana, reported a lower sensitivity (50%) (204), possibly due to difference in strain type (most other studies were performed in Asia) or technical factors that have since improved. The CELISA (*W. bancrofti* and *Brugia* spp.) and Brugia Rapid (*Brugia* spp.) tests performed on DBS eluate and compared with serum or plasma proved reasonably sensitive (71-98%) (99, 205). Cross-reactivity with other common parasitic infections, including *Onchocerciasis, Strongyloides, Ascaris* and *Dirofilaria* species are important (99, 205). Nucleic acid testing was evaluated for Brugian filariasis and *Loa loa* and appears sensitive, particularly for the latter at 96% (206-208). African and American trypanosomiases have both been successfully detected on DBS with high sensitivity and specificity by serology (209-212). A recent evaluation using a commercially available test for *T. cruzi* was, however, performed for relatively few patients (211). Strict control of humidity by storing DBS in sealed plastic bags with silica gel immediately after drying may have been a key factor resulting in the higher sensitivity reported by Chappuis *et al.* compared to Truc *et al.* (209, 210).

PCR testing on DBS for visceral leishmaniasis (*L. infantum*) in immunocompromised patients before therapy was evaluated against bone marrow microscopy in a small series of patients, yielding a sensitivity of 75% (213). PCR on DBS was significantly more sensitive than microscopy and culture of peripheral blood. The authors suggest a possible role for PCR as an initial screening test using DBS, potentially avoiding the more invasive bone marrow aspiration. Seroprevalence studies for echinococcosis, fascioliasis, and toxoplasmosis performed well on DBS. Detection of exposure to giardiasis suffered from lower specificity, possibly reflecting cross-reactivity or long-term persistence of antibodies (214, 215).

Table 2.8: Summary of studies evaluating DBS for parasites other than malaria.

Disease, assay type & country	Author	Number of samples & filter paper type	Test	Sensitivity %	Specificity %	Notes
Lymphatic Filariasis: Wucheraria bancrofti (Wb) Brugia malayi & timori (Bspp)Serology India(216)	Santhanam et al. (1989) (216)	94 samples Whatman 903	In-house EIA(217)	92	77	
Ghana	Gyapong et al. (1992) (204)	1808 samples Og4C3 paper	Og4C3 ELISA (Wb) (Tropical Biotech)	50.3	96.4	
Sri Lanka	Itoh et al. (1998)(201)	60 samples Nobotu 1	Og4C3 ELISA (Wb) (Tropical Biotech)	97	NR	
India	Hoti et al. (2002)(202)	30 samples Whatman no.3	Og4C3 ELISA (Wb) (Tropical Biotech)	76.6-93.3	100	Time of the day at which samples are collected impacts sensitivity (Hoti et al.)
India, Egypt, Haiti Kenya, Papua New Guinea, Sri Lanka	Wattal et al. (2007)(203)	188 samples Whatman no.3	Og4C3 ELISA (Wb) (Tropical Biotech)	NR	NR	
Egypt	Weil et al. (2011)(99)	81 samples TropBio paper	Filariasis (Wb & B.spp) CELISA (Cellabs)	91 (Wb) 98 (B. spp)	NR NR	Weil et al. based on a panel of known positives.
Uganda	Fischer et al. (2005)(205)	66 samples Whatman 3MM	Brugia Rapid (Malaysian BioDiagnostics Research)	79	NR	Significant cross-reactivity with other filarial infections(99, 205)

Disease, assay type & country	Author	Number of samples & filter paper type	Test	Sensitivity %	Specificity %	Notes
Lymphatic Filariasis *Brugia malayi* *NAAT* Indonesia(206)	Kluber et al. (2001)(206)	36 samples Whatman 3MM	In-house PCR(218, 219)	86	NR	
Malaysia	Rahmah et al. (2010)(207)	21samples Whatman 3MM	In-house PCR(218, 219)	NR	NR	
Loa loa *NAAT* Cameroon	Fink et al. (2011)(208)	68 samples NR	In-house PCR(208)	96	NR	No cross-reactivity with other filarial species. Limit of detection 1 microfilaria per 20uL DBS.
HAT *Serology (Card Agglutination Test)* Sudan(209)	Chappuis et al. (2002) (209)	100 samples NR	Micro-CATT (ITM Antwerp)	91	NR	
Central African Republic Ivory Coast	Truc et al. (2002)(210)	940 samples Whatman No. 4	NR	89.4-95.5	95.5-96.6	Truc et al. report rapid drop in sensitivity (67.8%) after 3 days without strict humidity control of paper. Ranges reported by Truc et al. reflect testing at 2 different sites.

Disease, assay type & country	Author	Number of samples & filter paper type	Test	Sensitivity %	Specificity %	Notes
Chagas disease Serology Brazil(211)	Luquetti et al. (2003)(211)	24 samples NR	Chagas Stat-Pak (ICT) (chembio Diagnostic systems)	100 ELISA 78.1	100 ELISA 99.7	Chagas Stat-Pak performed on small sample size (24). More sensitive and specific than large-scale evaluation with serum.
Brazil	Zicker et al. (1990)(212)	6222 samples Whatman No.1	In-house ELISA, IF & HA(220, 221)	IF 69.2 HA 64.6	IF 99.4 HA 99.6	
Echinococcosis Serology Argentina(222)	Coltorti et al. (1988)(222)	479 samples Whatman No.1	In-house ELISA	NR	NR	Coltorti reports sensitivity of DBS similar to that of serum.
China	Bartholomot et al. (2002) (223)	2482 samples Whatman No.1	In-house ELISA	96	87	
Uruguay	Cohen et al. (1998)(224)	1149 samples Whatman No.1	In-house ELISA	NR	NR	
Visceral Leishmaniasis NAAT Portugal	Campino et al. (2000) (213)	24 samples Whatman No.2	In-house PCR	71-75	NR	15/20 positive for patients not on treatment and 17/24 if those on treatment included. Useful as an initial screening tool.
Fascioliasis Serology Bolivia	Strauss et al. (1999)(225)	68 samples Whatman No.1	In-house ELISA	NR	NR	Samples stored for 10 years at 4°C were successfully detected.

Disease, assay type & country	Author	Number of samples & filter paper type	Test	Sensitivity %	Specificity %	Notes
Giardiasis Serology (IgG) Brazil(214)	Guimaraes et al. (2002) (214)	133 samples Whatman No.1	In-house IF	82	70	Guimaraes high rate of false positives with ELISA.
Saudi Arabia	Al-Tukhi et al. (1993)(215)	147 samples Whatman No.4	In-house ELISA	72-96	39-98	Al-Tukhi et al. results ranges depended on ELISA Optical Density reading and final eluate dilution.
Cysticercosis Serology Brazil(226)	Peralta et al. (2001)(226)	151 samples Whatman No.4	Qualicode Cysticercosis ELISA kit (Immunetics inc.)	80	NR	Good agreement between serum and DBS. May be useful initial screening test. All forms of neurocysticercosis stored for 1 week before freezing. Ranges due to samples processed at 2 sites using 2 methods.
Mexico	Fleury et al (2001)(227)	305 samples Whatman No. 311	In-house ELISA(228, 229)	39-66	87-96	
Toxoplasmosis Serology (latex agglutination) UK(230)	Parker et al. (1992)(230)	273 samples Whatman 903	Eiken Toxoreagent Latex Agglutination	98.8	100	
USA	Stevens etal. (1992)(231)	1098 samples Whatman 903	In-house ELISA	96	94	

2.7.1.7 BACTERIA

There have been suprisingly few studies evaluating the use of filter paper to diagnose or determine the seroprevalence of bacterial pathogens compared with viruses and parasites (Table 2.9).

The success of using DBS to screen for leprosy appears to be dependent on the bacillary burden, with multibacillary patients more readily identified (232-234). The commercially available Serodia Leprae particle agglutination test (Fujirebio, Japan) using DBS had 97.5% concordance with serum for patients of any bacillary burden type (235). Interestingly, the sensitivity of capillary DBS taken from the earlobe was slightly but significantly higher when compared to venous DBS and serum, possibly reflecting the higher density of organisms in this tissue (232).

Brucella antibodies were eluted from filter paper with difficulty and correlation coefficients with serum were modest, suggesting only a possible role in screening for cases (236). However, correlation coefficients are not valid statistical tests for comparison of diagnostic methods (bland reference). Other bacterial pathogens performed well on DBS and could be stored successfully for sufficient periods of time to allow transport to a laboratory for analysis (237-239).

Table 2.9: Summary of studies evaluating DBS with serological assays for bacteria

Disease, assay type & country	Author	Number of samples & filter paper type	Test	Sensitivity %	Specificity %	Notes
Leprosy Serology Nepal(232)	Butlin et al. (1997) (232)	200 samples NR	In-house ELISA(240)	NR	NR	Earlobe capillary blood more sensitive than serum or fingerprick blood.(232)
French	Chanteau et al. (1989)(233)	168 samples Whatman No.1	In-house ELISA	Multibacillary 96 Paucibacillary 29	Multibacillary 96 Paucibacillary 96	
Polynesia	Dhandayuthapani et al. (1989)(234)	94 samples Whatman No.3	In-house ELISA	NR	NR	
India	Sekar et al. (1992) (235)	81 samples Whatman No.3	MLPA (Fujirebio, Japan) In-house ELISA	67.7 (MLPA) 76.9 (ELISA) NR	98.7 (MLPA) 83.4 (ELISA) NR	
Orientia tsutsugamushi and Rickettsia typhi (Scrub & Murine typhus) Serology Laos(237)	Phetsouvanh et al. (2009)(237)	53 scrub samples 53 murine samples Whatman 903	In-house ELISA	95 IgM 90 IgG 91 IgM 82 IgG	88 IgM 100 IgG 100 IgM 100 IgG	Lower antibody titres with DBS. Storage at room temperature for 1 month did not affect antibody titres.(237, 238)
France	Fenollar et al. (1999)(238)	94 samples Fischer Scientific paper(238)	In-house ELISA(241)	100	100	

Disease, assay type & country	Author	Number of samples & filter paper type	Test	Sensitivity %	Specificity %	Notes
Leptospirosis Serology (MAT) La Reunion	Desvars et al. (2011)(242)	52 samples Whatman 903	MAT -Microscopic Agglutination Test	100	100	DBS samples showed lower antibody titres compared with serum.
Syphilis Serology Tanzania	Coates et al. (1998) (243)	1037 samples Whatman 903	Serodia TPPA (Fujirebio)	98.3	100	
Endemic syphilis (Yaws) Serology Papua New Guinea	Backhouse et al. (1992)(239)	70 samples Whatman 903	TPHA - Serodia TP kit (Fujirebio)	96.5	100	Results unaffected by up to 2 months storage.
Brucella Serology Spain	Takkouche et al. (1995)(236)	160 samples Whatman 2992	Brucella ELISA (Virotech System Diagnostika)	NR	NR	Pearson correlation coefficient: r = 0.8 for IgM and IgG. Time consuming extraction method.

2.7.1.8 Use of filter paper for samples other than whole blood

Filter paper has been used to store almost any clinical specimen for subsequent analysis. Whole blood is the most practical sample to collect on paper, however many reference assays use other samples types (e.g. serum or plasma) and some diseases are preferably diagnosed using other specimen types. Here we provide an overview of these specimen types. For full details see Annex 10.2.6.

Evaluation of dried serum spots to detect Hepatitis A antibodies showed a sensitivity and specificity of 100% compared to liquid serum (244) and HIV ELISA a sensitivity of 83% (245). NAATs perform very well with Hepatitis A (92.3% and 100%) and HCV (100% and 100%) sensitivity and specificity respectively (244, 246). Both hepatitis viruses showed a 10-fold fall in viral load after storage for 4 weeks on filter paper at room temperature (244, 246).

Three studies used dried plasma and one, dried breast milk, compared to liquid plasma for HIV quantitative PCR (247-249). HIV RNA on filter paper was stable at room temperature for > 1 year. Buffy coat may be used as a substrate to detect HIV proviral DNA. When dried buffy coat spots were compared with liquid samples, there was 100% concordance between results (250).

Although bone marrow is a difficult sample type to obtain, it is the most sensitive substrate for diagnosis of visceral leishmaniasis. In one small study, 34 out of 35 patients with diagnosis based on clinical presentation, was detected by NAAT on dried bone marrow spots. This was more sensitive than microscopy (251).

Cutaneous and mucocutaneous samples may be scraped, aspirated or directly impressed onto filter paper to diagnose leishmaniasis and, using slit skin smears, leprosy. The sensitivity cutaneous impressed filter paper for leishmaniasis ranged from 92.3-100% and specificity 100% compared to PCR on tissue samples (252, 253) and parasite speciation was possible. *M. leprae* was detected by PCR from dried slit skin smear spots (60%) in patients with known leprosy as frequently as the standard technique of skin slit smear storage in 70% ethanol (58%) (254).

Sputum and saliva have been more widely examined. HIV PCR on saliva had a low sensitivity when tested on viraemic patients with known HIV (255). Only 67% of serologically positive measles patients were positive by PCR on dried saliva spots, which was inferior to whole saliva and throat swab (256). Detection of malaria DNA in dried saliva (and dried urine) spots were less sensitive than blood microscopy (257). Dried induced sputum spots and dried bronchoalveolar lavage fluid spots to identify *Pneumocystis carinii* by PCR was reported with sensitivity of 67% and 90-91%, respectively compared to microscopic examination of these samples (258).

Dried cervical fluid spots were evaluated for Human Papilloma Virus by PCR. Concordance of 94-100% was reported in two of three studies compared with PCR directly on smear or cytobrush samples (259-261).

Cerebrospinal fluid (CSF) in children with meningitis was dried onto filter paper (dried CSF spots) and assayed by PCR for *Streptococcus pneumoniae* and *Haemophilus influenzae* with a sensitivity of 92% and 70% and specificity of 99% and 100%, respectively, compared with direct CSF PCR (262). The detection of neurocysticercosis IgM antibodies was less successful, ranging from 52-63%, in comparison to CSF, depending on the type of filter paper used to store CSF (227).

Both stool and urine have been stored on paper. *Vibrio cholerae* the organism could be cultured from dried stool spots after 14 days if humid conditions were maintained (263) and was equivalent to standard transport medium. Viral enteric pathogens including Norovirus, Rotavirus and Adenovirus serotypes 40 and 41 were detected by NAAT from dried stool spots. There was good concordance with EIA performed directly on stool (264-266). Pre-treating the paper with SDS/EDTA inactivated the virus, allowing safe handling of the paper. CMV is readily detected in urine in viraemic patients. Dried urine spots were reported to have 90% concordance with PCR on DNA extracted directly from urine(267).

2.7.1.9 Use of filter paper in veterinary health

Filter paper has been widely used in veterinary health, both in livestock and wildlife diseases as a specimen substrate. Several zoonotic diseases discussed above, including echinococcosis, brucella and trypanosomiasis (268) are also important causes of mortality in animals. However, non-zoonotic diseases are responsible for about half of livestock losses worldwide (268). Poultry, swine and cattle suffer the greatest burden of disease, with viruses and parasites the major causes of these often-fatal infections.

Severe commercial losses occur in livestock industry and global surveillance and diagnosis is necessary to collect information on ecology and install early warning systems to detect highly pathogenic strains such as Avian Influenza virus (AIV). The difficulties of traditional sample collection methods, discussed above for humans, are equally applicable in the veterinary setting. Filter paper has played a key role in circumventing many of these challenges for veterinary medicine. Smith *et al.* discuss the problems likely to be faced with the use of (FTA) filter paper with veterinary samples (269).

Porcine reproductive and respiratory syndrome viruses (154), Newcastle disease, AIV and *Theileria* species (270, 271) have been successfully identified from blood DBS. Samples other than blood are perhaps more frequently encountered in animal, than human, disease diagnosis. Examples include cloacal swabs adsorbed onto paper to detect AIV (272), bursal fluid to identify infectious bursal disease virus of poultry (273), tongue epithelial samples from cattle for foot and mouth disease virus (274) and canid brain tissue to identify rabies (275). These methods are also applicable to wildlife as demonstrated by Curry *et al.* who identified Brucella antibodies in dried caribou blood with high sensitivity and specificity (276). Leishmaniasis is an important zoonosis with reservoirs in canids. However serological studies amongst dogs using filter paper compared with serum have given relatively poor sensitivity of 22.2% or an agreement of 68.8 % ($k = 0.234$) (277, 278) FTA paper carries the advantage of inactivating highly pathogenic organisms to allow safe transportation with reported complete inactivation of highly pathogenic AIV one hour after following adsorption onto FTA paper (279).

Human and animal health are inextricably linked but there has been very little, if any, collaboration between scientists and health workers interested in human and non-human health and filter paper diagnostics. More 'One Health' collaboration on these techniques would benefit both fields.

2.8 Discussion

Over the last 50 years, filter paper has gained an increasingly important role as a substrate for diagnosis, epidemiology and surveillance of infectious diseases. Recently, this role has gone beyond diagnosis to include detection of markers of resistance, detailed genetic or serological analysis and the monitoring of therapeutic interventions including drug levels, vaccine-induced responses and viral loads.

Almost any clinical sample may be stored on filter paper for subsequent analysis, though finger-prick blood is the most convenient and widely used.

Viruses, particularly HIV, have been most frequently targeted with filter paper diagnostics. Serological tests perform very well with seven studies reporting sensitivity and specificity close to 100%. NAAT performance is more variable due to the greater instability of nucleic acids, but mostly reached similar accuracy. Infant diagnosis using both RNA and DNA are feasible, however RNA tests tend to suffer with reduced specificity. Hepatitis viruses, many of the Herpes virus family, measles, and rubella also perform well with serological tests with sensitivities and specificities of >90%. NAATs appear promising, though more evaluations are needed particularly for HCV and HEV. Dengue serology performed on DBS is clearly suitable for seroprevalence studies, though this is less clear for the diagnosis of acute primary and acute secondary infections. A recent surveillance study suggests that including dengue NS1 detection with anti-dengue IgM may increase sensitivity (280). Dengue serotyping is epidemiologically important and can also successfully be performed using DBS (165, 280).

DBS also play a key role in the diagnosis of parasitic infections. Detection of malaria by PCR using in-house methods is generally superior to the gold standard of microscopy. Most studies report sensitivities of >94% and specificities of >99% (182, 183, 196, 198). Due to the geographical distribution of filariasis and its prevalence in remote settings, filter paper has been extensively used to diagnose patients, determine epidemiology and monitor response to eradication programs. Using commercially available assays sensitivities of >90% may be achieved (99, 201). Specificity is more varia-

ble due to problems associated with cross-reaction with other related parasites (99, 205). Leishmanisasis, giardiasis and cysticercosis have proved less promising in the few studies that have evaluated DBS compared to a recognized gold standard (213, 214, 227).

The detection of bacteria has less often been investigated using filter paper. Serological tests for leptospirosis, treponema infections and rickettsia have yielded excellent results (238, 242, 243), whilst others, such as leprosy, have been less successful (235). The selection of pathogens that may perform well on filter paper is dependent on several important factors. The presence and quantity of serological markers and nucleic acids in the blood at the time of sample collection, their stability on filter paper and the development of sensitive and specific assays adapted to perform on filter paper.

There are several key advantages of using filter paper over the traditional specimens of whole blood or serum. Many of the pathogens discussed above are most common in remote and resource-poor settings with limited access to advanced diagnostic facilities. Filter paper obviates the need for a cold chain to preserve specimens in transport to a central laboratory, thus enormously increasing accessibility of these tests worldwide. Filter paper is generally cheap (although some of the treated papers, such as FTA, are very expensive), requires only a small sample volume (which may be more acceptable to the patient), less of a burden for the health system and needs minimal technical expertise to perform. This is likely to make sample collection more acceptable to the patient and increase testing uptake (281). Filter paper is easily and safely delivered using almost any existing transportation network available. Recent advances in chemically pre-treated cards have provided increased safety in handling and transporting samples (279). Perforated cards allow simpler and quicker preparation of samples for the elution step, limiting the need for punching tools and reducing cross-contamination risks. Samples are stored and transported at ambient temperatures and can usually be stored for prolonged periods if sufficient precautions against humidity are taken. Filter paper has been used with multiplex serological and NAATs to diagnose combinations of Hepatitis B, C and HIV (282, 283). This will increase the diagnostic potential of a single dried blood spot.

There are, of course, important difficulties and limitations to be encountered when using filter papers. It was apparent when conducting the literature search and review that a great variety of terminology has been employed when discussing filter paper. Studies evaluating the same pathogen often use different methodologies encompassing almost every stage of the process from filter paper selection to final assay procedures, making comparison vexed. Some studies have used DBS without a justification that the method is accurate against a reference standard. Many filter paper varieties have been used (products are not always clearly labelled with the paper weight in g/m^2) and sample volumes will vary, so care is advised when moving between paper types. A consensus document on terminology and methodology would be invaluable for advancing the field of filter paper diagnostics.

The importance of standardized sample collection, storage and processing of filter paper and justification of the accuracy of the methodology in comparison to conventional techniques cannot be overstated. High temperatures and humidity over prolonged periods severely reduce test sensitivity particularly for NAATs, though this seems to vary between pathogens (137, 138, 141, 159, 169). Inevitably the sample size of DBS – the volume of blood per spot – will be less than that of a whole blood sample collected by venipuncture. DBS containing whole blood may also influence NAATs or serological assays due to the presence of inhibitors. These can, however, be overcome by developing DBS specific protocols (284, 285). Although some guidelines exist, there is an urgent need for more robust, standardized protocols for sampling, storage, processing and evaluating filter paper techniques. Many studies reported in this review were not prospective, real life evaluation such studies would provide a stronger evidence base to support recommendations. Additionally, most studies used pipettes to spot venous blood onto filter paper which gives a greater consistency in blood volume than direct application of blood to paper, that is unlikely to be achieved with field samples. A number of studies did not report sensitivity and specificity and several inappropriately used correlation coefficients rather than Bland-Altman plots (168). The standards for the reporting of diagnostic accuracy studies (STARD) guidelines are an important starting point for reporting filter paper evaluation (286). The inclusion of additional items to improve accuracy and

completeness of filter paper studies (Annex 10.2.7), could greatly improve consistency and clinical utility of the results. In brief, these could include the use of standardized terminology for DBS, sample collection, storage and processing details, key information regarding the reference standard and the use of appropriate statistical tests (e.g. Bland Altman plots versus correlation coefficients).

The review has important limitations. First, in attempting to summarize studies where DBS were evaluated in comparison to a gold standard to diagnose infectious diseases, we did not include related subjects such as drug resistance and viral load. Second, we excluded in-house assays for those diseases with well-recognized commercially available assays though these are listed in annex 10.2.8. Third, we did not do a detailed assessment of veterinary use of filter paper, as this subject would require an independent literature review in its own right. This highlights the need for more collaboration between scientists in human and animal health. Fourth, we were only able to review studies published in English.

This is the first attempt to summarize the entire subject of filter paper diagnostics in infectious diseases. We highlight the many advantages filter paper offers over traditional samples and discuss the limitations and difficulties associated with using this method of storing and processing clinical samples. Consensus should be reached regarding the methodology and terminology employed to better advance this important diagnostic tool. Filter paper has been shown to be a valuable asset in increasing accessibility of infectious disease diagnostics. In some cases, the sensitivity and specificity is slightly lower than the reference sample, however it is the potential of filter paper to make affordable, robust, sensitive, and specific diagnostics available to remote settings that raises its profile in international health.

2.8.1 Advantages and disadvantages of DBS

As practically every aspect of DBS has been described and evaluated in this chapter, Table 2.10 summarises the main advantages and disadvantages of DBS compared to venous blood collection by venipuncture.

Table 2.10: Advantages and disadvantages of DBS compared to venous blood collected by venipuncture

Advantages of DBS	Disadvantages of DBS
Improves access to diagnostics	Small volume of blood affects sensitivity
Lower costs	Contamination risks affects specificity
Ease of collection	Extraction of DNA/RNA is more complicated
Transportation - no cold chain needed	Adjustments required to protocols/ assays
Small quantity of blood collected	
Better participation in studies	
Less biological waste produced	
Small size – less storage space required	
Minor patient discomfort	

2.8.2 Recommendations

The STARD initiative was launched in 2003 to develop guidelines for improving the accuracy and completeness of reporting studies of diagnostic accuracy (286). During the literature review, we encountered a lack of accuracy and incompleteness in reporting findings, even sensitivity and specificity were not always reported. Based on the key issues encountered when reviewing the literature of DBS evaluations, the STARD checklist was amended to cover the specific details that should be reported when evaluating DBS samples. The key issues were combined with the additional points to the STARD checklist in Table 2.11. The complete STARD checklist, with the amendments highlighted, can be found in Annex 10.2.7.

Table 2.11: Summary of key concerns in reporting DBS studies and added STARD checklist points

Concerns in reporting DBS studies	STARD checklist adjustments for DBS evaluations
Inconsistency in terminology	Make use of terminology: i.e. Dried Blood Spots, Dried Urine Spots, Dried Fluid Spots, Dried "Samples" Spots
Unclear or not reporting filter paper sample collection method.	Sample collection: State which filter paper was used, which and how fluids were obtained and spotted onto filter paper, and the drying period before storage
Unclear reporting of reference method and sample.	Report the index sample and its collection, storage, and transportation details. Provide detailed rationale for discordances in methods between index and reference test.
Unclear or not reporting storage and time between collecting and analysing samples.	Sample processing: state the time and storage conditions (humidity control and temperature) at the field, during transportation, and at the laboratory, preferably in a tabled manner.
Unclear or not reporting punch method and cleaning procedure.	Report punching method with reference to source or manufacturer, and cleaning procedure, if used.
Unclear or not reporting how quantitative data was obtained from filter paper samples.	For quantitative or numerical test results, indicate the calculation methods and rationale of the index and reference standard
Unclear or not reporting the biological variability of samples and mean difference between index and reference sample	For quantitative test outcomes, report the mean and range of results for index and reference test
Unclear or not reporting of diagnostic accuracy of quantitative test outcomes.	For quantitative test outcomes, Estimates of diagnostic accuracy and measures of statistical uncertainty (e.g. 95% confidence intervals) by quantitative grouped ranges (e.g. 1,000-5,000 copies/mL).

Systematic review of DBS in HIV viral load patient monitoring and early HIV infant diagnosis

Chapter 3

3 Systematic review of DBS in HIV viral load patient monitoring and early HIV infant diagnosis

3.1 Introduction to publication

Access to antiretroviral therapy (ART) has increased over the last few years, with over eight million people receiving ART in middle and low income countries, in 2011 (287). CD4 cell counts aid clinicians when to initiate therapy and HIV VL aid to decide when therapy is failing. Access to these tests remains limited due to high costs, necessary laboratory infrastructure, and technical complexities in developing countries (66). When these tests are not available, treatment failure is based on clinical or immunological criteria, leading to undesirable late identification and accumulation of resistance mutations (288). Additionally, these tests may be used to detect acute HIV-1 infection or infant diagnosis.

To monitor treatment efficacy and to guide clinical decision making for switching treatment therapy in developing countries, there is an urgent need to evaluate simplified methods that can improve accessibility of patient monitoring assays (36, 289). Additionally, access to HIV VL testing and EID is limited to the population living in close proximity to the laboratory because of the need for venous blood samples. To answer this need, this Chapter assessed the use of DBS for HIV VL and EID and potentially to improve accessibility to these tests in remote settings.

3.2 Research paper

Systematic review of the use of dried blood spots in HIV viral load patient monitoring and early infant diagnosis

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Status: Not yet submitted

Contributions: PWS initiated this study to review the use of DBS for HIV VL, as part of the study request made by the WHO to perform a systematic literature review for HIV VL assays performed with plasma samples. PWS and KS performed the systematic literature search and extracted data. PWS wrote the first drafts of the manuscript and KS provided comments and feedback. RWP provided guidance during the whole process and contributed significantly to the writing of the manuscript. HIV Monitoring Technologies Working Group has not read this or an earlier version of the manuscript yet.

The candidate The supervisor

Abstract

The importance of adequate HIV viral load monitoring and early infant diagnosis in remote settings is rising, given that access to and the use of treatment is rapidly increasing. Dried Blood Spots (DBS) have been considered as valid alternative to increase access to these tests in remote settings. We therefore provide an overview of the methods and identify gaps that need to be sorted before DBS can be widely used as the preferred sample choice in remote settings. Thirteen HIV viral load articles and six HIV early infant diagnosis papers were included. Although some challenges surrounding the pre-extraction and analytical stages need to be resolved, DBS can be used as an alternative to plasma for HIV viral load quantitation based on the suggested threshold of 5000 c/ml as cut-off for treatment failure. Although limited data is available for infant diagnosis, DBS seems a very sensitive and specific sampling strategy to make diagnosis more accessible.

Introduction

According to the latest WHO figures, in 2011 34 million people were living with HIV worldwide. Young women aged 15-24 are most vulnerable to become HIV positive, as infection rates are twice as high as men of the same age. This puts children at risk as well, and in 2009, 370.000 children were born HIV positive (290).

Over eight million HIV infected individuals have been placed on antiretroviral therapy (ART) and will require on-going monitoring to ensure treatment continues to be efficacious (287). HIV VL testing is an important tool for monitoring treatment, detecting treatment failure and preventing misclassification of treatment responses and inappropriate switching of treatment regimens (291).

In many resource-limited countries, it is a challenge to diagnose HIV infected infants because antibody diagnostic assays cannot be used. As HIV positive mothers pass IgG antibodies on to the infant while in the womb, antibody tests can only accurately be used when the child reaches at least 18 months

of age but by this time, infants may be lost to follow-up and their infections remain un-diagnosed untl they present clinically at a health care facility. Early infant diagnosis (EID) relies on the detection of HIV nucleic acids to confirm HIV positivity of the infant.

Assays to detect HIV nucleic acids are more technically advanced and costly than simple antibody tests and as a result, EID and VL testing are not readily available in primary healthcare facilities. Although used for different diagnostic purposes, the problems in access to EID and HIV VL testing in low income countries are the same. Both tests are often not performed because of the costs, and requirement for expensive laboratory equipment and trained technicians (292, 293). Additionally, access to HIV VL testing and EID is limited to the population living in close proximity to the laboratory because of the need for venous blood samples.

Alternative sampling methods have been reviewed as a way to increase access to these tests in remote areas (38, 66, 294, 295). The use of filter paper for the collection of whole blood, Dried Blood Spots (DBS), has several advantages over traditional methods of sample collection. DBS can be implemented as an easy and inexpensive means of collecting and storing blood specimens under field conditions (30-34). The reduction in materials required, and biological waste produced, as well as the ability to collect sample by heel or finger prick without phlebotomy , considerably decreases costs for sample collection, compared to venipuncture (35). DBS also increases accessibility of HIV VL and EID testing in remote areas (36, 37).

The process of DBS collection begins with a finger prick and spotting whole blood directly onto filter paper which is then left to dry at room temperature. Once dried, DBS can be stored with desiccant and shipped to central laboratories for HIV VL testing. The effects of using an alternative sampling method for HIV VL and EIA assays should be well understood as this potentially influences the sensitivity and accuracy (296).

Since both tests are performed on comparable laboratory equipment that detects nucleic acids, we performed a literature review to assess the feasibility of using DBS samples for HIV VL and EID tests. Although literature re-

views have been published before that reviewed the feasibility of using DBS for HIV VL (36, 55, 297), this literature review provides an updated technical analysis of commercially available platforms and the use of DBS samples for HIV VL and EID. This review was initiated to critically assess the differences between DBS and plasma HIV VL measurements on commercially available HIV VL assays. Additionally, no literature review has been published yet that reviewed HIV EID with DBS samples.

Material & methods

We performed a systematic review of studies evaluating the use of DBS for HIV viral load quantification and for EID. The search protocol is given in Annex 10.3.1.

Eligibility criteria

Eligibility criteria were defined using PICOS (Population, Interventions, Comparisons, Outcomes, Study Design). Studies evaluating DBS and plasma samples for HIV viral load measures with commercially available technologies at the time of the review (April 2012) were considered for inclusion.

PICOS HIV VL DBS

Participants: HIV positive persons from any geographical location.

Intervention (Diagnostic Assay): Any commercial technology available for the quantification of plasma HIV-1 viral load at the time of the search with Dried Blood Spot samples.

Comparators (Reference Standard): Plasma samples

Outcomes: Evaluations or comparisons of the accuracy and/or reproducibility of the index sample type.

Study Design: Evaluative studies using an acceptable reference technology or comparative studies.

Other: Scientific articles published in peer reviewed journals, in English, from 1990 to present.

PICOS EID DBS

Participants: infants from any geographical location.

Intervention (Diagnostic Assay): Any commercial technology available for the detection of HIV-1 DNA or RNA at the time of the search with Dried Blood Spot samples.

Comparators (Reference Standard): whole blood, plasma, or serum samples

Outcomes: Evaluations or comparisons of the accuracy and/or reproducibility of the index sample type.

Study Design: Evaluative studies using an acceptable reference technology or comparative studies.

Other: Scientific articles published in peer reviewed journals, in English, from 1990 to present.

Information Sources

Studies were identified by searching the electronic databases MEDLINE and Embase, and by inviting experts from the advisory group (HIV working group) to identify studies relevant to the review. The search was developed by PWS, KS and RWP, conducted by PWS and KS.

Search Strategy

The following search terms were used for HIV VL search: dried or dry, blood, spot, DBS, filter paper, Guthrie card, 903 paper, HIV, human immunodeficiency virus and human immune deficiency virus. The initial search for evaluations of DBS for HIV VL quantitation was conducted 15 February 2010, and updated 18 January 2012. For the search of publications evaluating DBS for EID, the following search terms were used; dried or dry, blood, spot, DBS, filter paper, Guthrie card, 903 paper, HIV, human immunodeficiency virus and human immune deficiency virus, DNA, RNA, infant, neonatal, neonate, early infant diagnosis, EID. This search was conducted 2 April 2012.

Study selection

Titles and abstracts were screened for relevance. The full text of eligible articles were reviewed and assessed against inclusion criteria. The inclusion criteria were:

- Evaluation or comparison of performance of commercially available viral load quantification assays or EID assays
- Evaluation of dried blood spots with valid reference sample using the same viral load or EID technology
- Any HIV-1 subgroup recognition
- Quantitation of plasma and dried blood spots for viral load and detection of RNA and/or DNA for EID

For HIV VL, 473 articles were retrieved, 459 were excluded, and 13 articles were included. For EID, 225 articles were retrieved, 220 were excluded and 4 articles were included (Annex 10.3.1).

Results

Early infant diagnosis

Five studies evaluated the performance of early infant diagnosis (EID) assays with DBS against whole blood or plasma samples as reference method using the same EID assay (144, 145, 298-300). Four studies used Whatman 903 filter paper (144, 298-300) and one study used Whatman No.1 filter paper (145). Out of the five studies, Amplicor 1.5 assay (n=4) and Cobas Taqman were evaluated. More details can be found in Appendix 3. The samples used for the evaluations were of infants younger than 18 months (144, 145, 298), 6 weeks old (299), and of children between 6 weeks and 6 years (300). The sensitivity and specificity of the various assays are given in Table 3.1.

Table 3.1: Sensitivity and specificity of HIV early infant diagnosis

Author	Assay	VL Threshold	Sample size	Sensitivity*	Specificity*
Anitha et al. (298)	Amplicor 1.5	NA	64	100	100
Lofgren et al. (144)	COBAS Taqman	>1.000	176	100	99
		>10.000	176	100	100
Nsojo et al. (145)	Amplicor 1.5	NA	325	100	99.6
Sherman et al. (299)	Amplicor 1.5	NA	2880	100	99.6
Stevens et al. (300)	Amplicor 1.5	NA	800	100	99
Stevens et al. (300)	COBAS Taqman	NA	800	100	100

*sensitivity and specificity of DBS calculated compared to whole blood or plasma

HIV VL

Thirteen studies evaluated the performance of quantitative HIV VL assays with DBS samples against plasma (38, 123, 289, 296, 301-309). All studies used Whatman filter paper 903 (protein saver card, GE healthcare, USA) (38, 123, 289, 296, 302-306, 308). Among the thirteen studies, the following platforms were used; Abbott RealTime HIV-1 assay performed on the M2000 platform (N=5), BioMerieux NucliSens easyQ v1.1 (N=1) and v1.2 (N=3), Roche COBAS Taqman HIV-1 viral load (N=2), Roche Amplicor Monitor v1.5 (N=2), Versant HIV-1 kPCR (N=1) (Table 1). DBS VL results were compared to plasma results on the same platform (38, 123, 289, 296, 302-306, 308). Ikomey et al. conducted plasma and DBS samples in one run, while all

remaining evaluations used two runs to quantitate HIV VL using DBS and plasma samples (302).

DBS preparation

DBS samples were prepared with either EDTA-blood obtained from a venipuncture (38, 289, 296, 301-306, 308) or heel prick blood (123). Sample storage conditions varied from -20°C (301), -70°C (123) to ambient temperatures (38, 305, 306, 308). Samples in eight studies were humidity controlled with desiccants (38, 123, 289, 301, 306, 308) but six studies did not specify if desiccants were used (296, 302-304). Plasma samples were stored at -80°C (38, 305, 306, 308), -70°C (123, 303), -20°C (296, 302) or not specified (304, 308). All studies included samples with a plasma HIV VL range starting from 2 or 3 $\log_{10}$ copies up to 7 $\log_{10}$ copies/mL. More study details can be found in Annex 10.3.3.

Extraction methods

The NucliSens extraction platforms were used by 6 studies. Andreotti *et al.* (289) began the extraction of DBS samples with the Roche automated viral isolation platform but continued to use the NucliSens isolation due to incorrect isolation of RNA specimens. The M2000 sample preparation platform (M2000sp) was used by 6 studies in combination with the M2000 detection platform.

Sample volume and haematocrit

The HIV VL measurements obtained by DBS samples, which roughly contain 50 - 100 µl of whole blood, were compared to plasma samples in thirteen studies. Plasma input volumes for the reference VL testing were 100 µl (303), 500 µl (289), 600 µl (305) or unspecified (38, 123, 296, 301, 302, 304, 306, 308). Because of this sample input difference, it is theoretically impossible that DBS and plasma samples give comparable viral load results. Marconi et al. found a mean difference between DBS and plasma VL of 1.94 ± 0.06 log10 copies/mL and provided this as a correction factor for DBS VL calculations (304). An Abbott RealTime HIV-1 assay protocol for DBS

samples is now available, incorporating this correction factor. Haematocrit values can be used in the viral load corrections by estimating the amount of plasma in a 50µl whole blood sample. One study found that haematocrit correction reduced the difference of DBS and plasma viral load from -0.43 log10, to -0.127 log10. (123). An alternative is to reduce the reference sample volume for a better VL correlation of DBS and plasma (303). 4 out of 13 studies reported that DBS VL were corrected for haematocrit or that DBS were corrected for the smaller sample input volume (123, 289, 304, 307).

Bias

The mean difference, bias, between DBS and plasma VL measurements ranged from -0.77 (HIV VL underestimation) (296) to 0.65 log10/mL (HIV VL overestimation) (301) across all studies (Figure 3.1). For the Abbott RealTime HIV-1 assay, 51.9% up to 100% of the DBS HIV VL results were within 0.5 log difference of plasma samples (38, 296, 301, 304, 305, 309). The percentage of samples within 0.5 log for the Roche COBAS Taqman HIV-1 viral load and Versant HIV-1 kPCR where 78.4% (289) and 82.7% (306), respectively. The BioMerieux NucliSens easyQ v1.1 was evaluated by two studies for which 64% (296) and 94% (303) of the results were within 0.5 log. The greatest variance between DBS and plasma was found in studies using the Abbott RealTime HIV-1 assay (Figure 3.1).

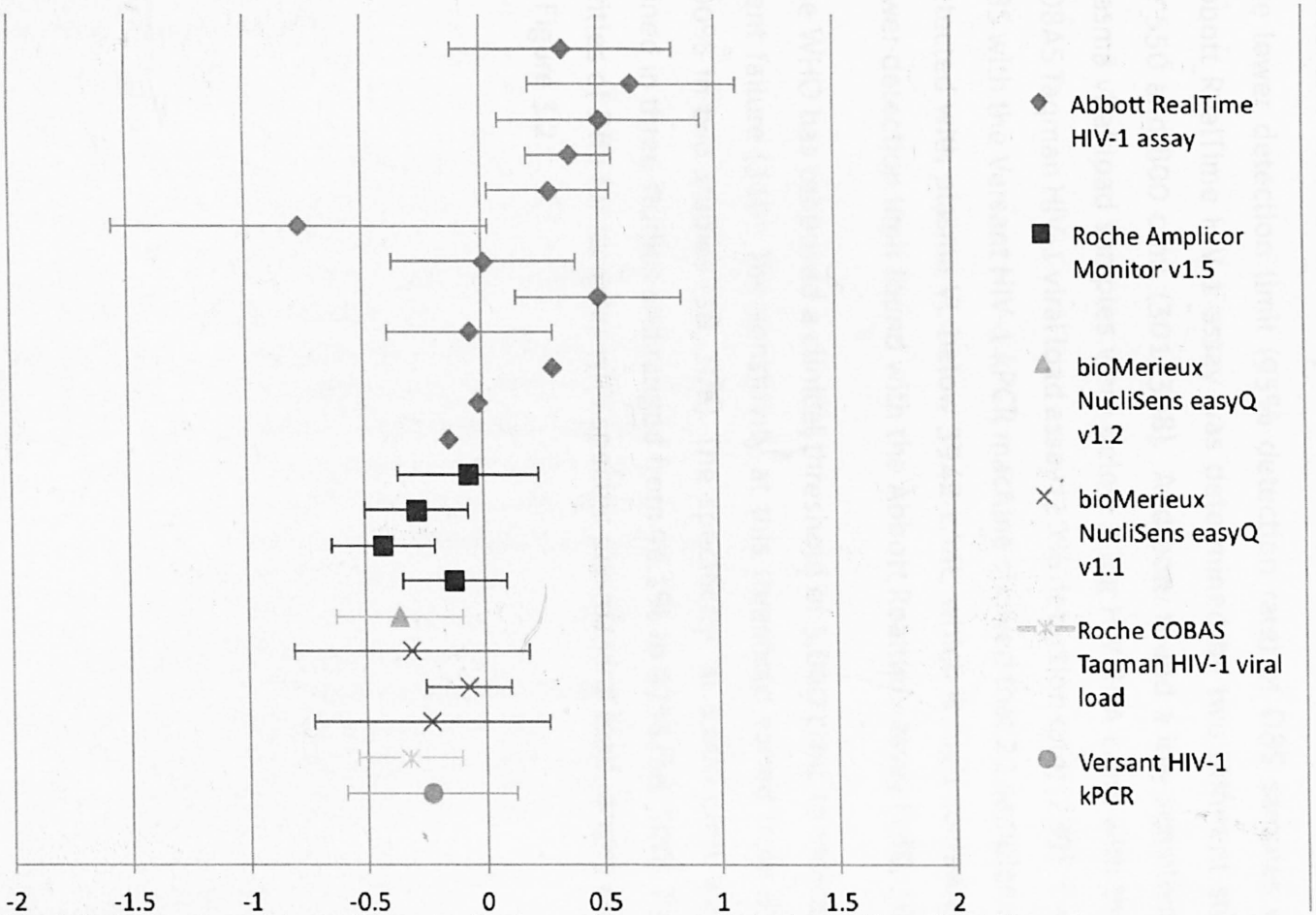
Table 3.2: Sensitivity and specificity given per VL threshold used

Author	Assay	VL Threshold	Sample size	Sensitivity %*	Specificity %*
Lofgren et al. (144)	RealTime	1000	176	100	99
		10000	176	100	100
		400	137	99	87
		5000	137	100	97
Pirillo et al. (306)	kPCR	37	98	88.2	69.2
		5000	98	85.1	96.1
Rottinghaus et al. (307)	NucliSens v.1.1	1000	173	77.8	98.1

* sensitivity and specificity of DBS calculated compared to whole blood or plasma

Figure 3.1: Bias per HIV viral load range, given for each assay.

HIV VL range	Reference
<40 - >100.000	Arredondo et al.
40-1000	Arredondo et al.
1000-5000	Arredondo et al.
5000-10.000	Arredondo et al.
10.000-100.000	Arredondo et al.
100-1.000.000*	Garrido et al.
200-100.000	Marconi et al.
200-1000	Marconi et al.
>1000	Marconi et al.
<3000	Vydia et al.
>3000 -1.000.000	Vydia et al.
200-1.000.000	Vydia et al.
400-800.000	Ikomey et al.
2201–6.450.490	Leelawiwat et al.
2201–6.450.490	Leelawiwat et al.
2201–6.450.490	Leelawiwat et al.
<100-1.000.000*	v Deursen et al.
100-1000000*	Garrido et al.
800-250.000	Kane et al.
250-2.500.000	Rottinghaus et al.
<100-1.000.000	Andreotti et al.
100-10.000.000*	Pirillo et al.



[a] haematocrit corrected, [b] viral load adjusted for smaller input volume, [c] standard haematocrit value used to correct, * data obtained from Bland-Altman figures .
Bias (DBS-plasma) was plotted as the mean with 1 standard deviation (SD). A negative value represents an HIV VL underestimation compared to plasma while a positive HIV VL bias represents an overestimation of the HIV VL compared to plasma. SD was recalculated to 1 SD when presented as 1.96 or 2SD. In case of discrepancy between bias reported in the text and Bland Altman figure, the bias noted in the text was used (301).

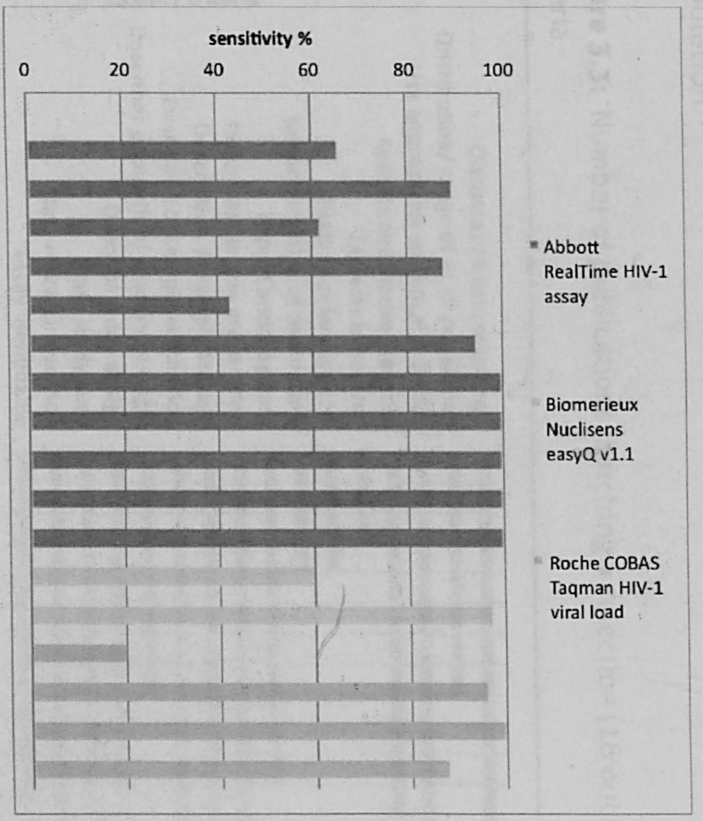
Detection limit and sensitivity

The lower detection limit (95% detection rate) of DBS samples with the Abbott RealTime HIV-1 assay was determined by two different studies to be 550 and 800 c/mL (301, 308). Andreotti found a low sensitivity when plasma viral load samples were below 3 log HIV RNA c/mL with the Roche COBAS Taqman HIV-1 viral load assay (20% detection rate) (289). Evaluating DBS with the Versant HIV-1 kPCR machine showed that 21 samples were not detected with plasma VL below 3942 c/mL, which is high compared to the lower detection limit found with the Abbott Realtime assay (289, 306).

The WHO has proposed a clinical threshold of 5,000 c/mL to indicate treatment failure (310). The sensitivity at this threshold varied from 85.1% to 100% in two studies (38, 306). The specificity at 5,000 c/mL was determined in three studies and ranged from 96.1% to 97% (38, 306). The sensitivities of DBS for samples with specific plasma viral load ranges are shown in Figure 3.2.

Figure 3.2: Sensitivity per viral load range of DBS compared to matching plasma samples

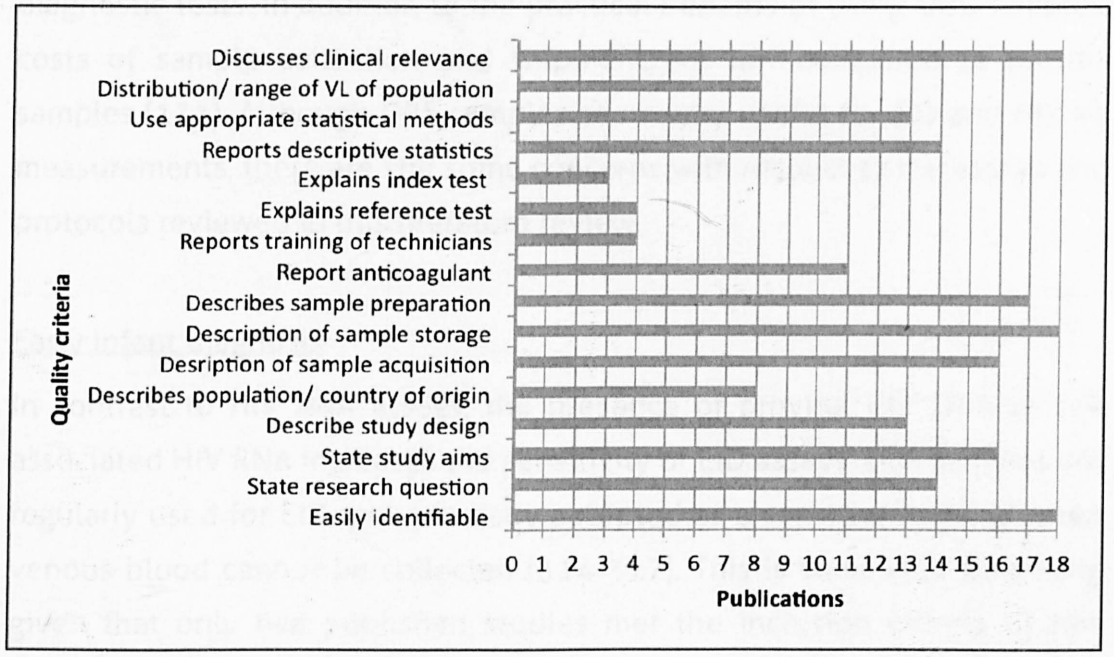
Reference	Viral load range range	Sample size
Mbida *et al* (305)	40-100	5
Mbida *et al* (305)	100-1000	3
Marconi *et al* (304)	200-1000	20
Vidya *et al* (309)	<1000	NA
Vidya *et al* (309)	1000-3000	NA
Garrido *et al* (296)	1700-5250	7
Marconi *et al* (304)	1000-10.000	57
Mbida *et al* (305)	1000-10.000	8
Garrido *et al* (296)	>5250	22
Marconi *et al* (304)	10.000-100.000	91
Mbida *et al* (305)	>10.000	19
Vidya *et al* (309)	>5000	NA
Garrido *et al* (296)	1200-3000	5
Garrido *et al* (296)	>3000	43
Andreotti *et al* (289)	45-1000	15
Andreotti *et al* (289)	1000-10.000	28
Andreotti *et al* (289)	>10.000	67
Andreotti *et al* (289)	45-10.000	110



Quality assessment

Each of the thirteen HIV viral load articles and 5 EID articles included in the review were assessed for quality by two independent reviewers. Twenty four criteria, based on the STARD guidelines, were used to assess the quality of publications (Figure 3.3). All 18 studies described sample storage conditions, discussed the clinical relevance and were easily identified as evaluation studies. 8 out of 18 studies described the population sampled and again only 8 studies described the distribution or range of HIV VL of the sampled population.

Figure 3.3: Number of publications matching a selection (18 out of 24) quality criteria.



Causes of variability in VL

All assays discussed in this literature review are sensitive enough to be used for DBS samples as the lower detection limit of each of these assays, according to the manufacturer, are below 100c/mL. The most important differences between the assays are input volume and the extraction method both of which contribute to differences in mean bias and variability. The method of punching out the filter paper spot, the punch size, elution buffer used as well as the sample volume after extraction that was used for VL measurement, alter HIV VL results. In the analytical stage, haematocrit correction, quantity of blood and quantity of plasma processed, viral load

calculation method applied, cell associated RNA as well as HIV proviral DNA can alter viral load measurements in some assays. When these critical phases in processing DBS are addressed and resolved, it will become possible to reduce result variances, develop uniform protocols which allow a more detailed comparison between sample collection methodologies.

Discussion

DBS samples have been used for many years in the detection of various infectious diseases, throughout the world (311-313). DBS samples have been used for diagnosis of HIV by antibody and antigen detection and have shown to be a very useful diagnostic tool to increase accessibility of diagnostic tests. In addition to the practical benefits of using DBS samples, costs of sample collection and shipment are low compared to plasma samples (131). Although DBS samples seem very useful for EID and HIV VL measurements, there are still some concerns with respect to the assays and protocols reviewed in this literature review.

Early infant diagnosis

In contrast to HIV RNA assays, the presence of proviral HIV DNA or cell associated HIV RNA increases the sensitivity of EID assays. DBS samples are regularly used for EID and generally accepted as a sensitive method when venous blood cannot be collected (314-317). This is somewhat surprising given that only five published studies met the inclusion criteria of this review for evaluation of DBS for EID. Although all studies showed good sensitivity, the specificity was slightly less than with liquid blood samples (99-100%). Clinically, this means that 1 in every hundred patients will be false positively diagnosed. Furthermore, only two of the 6 commercially available HIV platforms were evaluated. It is recommended that assays are thoroughly evaluated and the protocol validated before diagnosing patients using DBS samples.

Nucleic acid detection assays used for EID and HIV VL are often performed on the same or comparable platforms. While manufacturers provide separate kits for EID and VL, there is increasing interest to use HIV VL assays for

EID as well. HIV VL assays can detect HIV RNA and by using whole blood samples, DNA may also be detected which could increase sensitivity for EID. Additionally, using one platform and one kit for both purposes would potentially simplify testing procedures in the laboratory. Although more evaluations are needed, the use of HIV VL assays for EID with DBS samples could potentially decrease costs, improve EID accessibility and simplify testing procedures (148, 318).

HIV viral load

5000 c/mL threshold

Twelve out of thirteen studies suggested that HIV-1 VL quantification from DBS is feasible (38, 123, 289, 296, 301-306, 308). The sensitivity of DBS samples at a threshold of 5,000 c/mL, the suggested clinical threshold indicating treatment failure, varied from 85.1% to 100% in two studies. The specificity at 5,000 c/mL ranged from 96.1 to 97% (38, 306). Clinically, this means that 4 out of 100 HIV patients will switch treatment unnecessarily and 15 out of 100 will remain on the same treatment despite having elevated HIV VL levels. The difference between DBS and plasma samples could be the result of biological and assay variation, which could cause false treatment failure results.

The lower limit of detection in HIV VL quantitation assays when using DBS is 550-1000 c/mL, whereas with plasma, it may be as low as 200c/ml or even 20c/ml. One of the reasons DBS is not, and may never be, as sensitive as plasma in detecting low HIV VL, is that a complete DBS circle contains up to 50µl of whole blood which is approximately 20-25µl of plasma. The HIV quantitative assays are developed and validated for large plasma sample volumes (between 100 and 1mL) and calculate VL based on the expected plasma sample input volume. When a HIV VL platform has no specific DBS protocol, it is necessary to alter the HIV VL result measurement which was calculated by the software for plasma samples, and adjust it for the smaller volume of a DBS samples to obtain a final HIV VL result that would match a plasma sample.

Correcting HIV VL values

The DBS VL can be recalculated to HIV c/mL by applying the difference between plasma and DBS sample volume. To make the calculation, haematocrit values can be obtained to adjust DBS viral load results by calculating the amount of plasma in a DBS sample (123). Pirillo *et al.* made use of carefully designed reference standards for calculating DBS VL levels but unfortunately did not give details about the calculation. These calculations are important to compare DBS VL results between authors and to understand the effects of these adjustments on over- or under-quantification of HIV VL results. It is recommended to contact the HIV VL assay manufacturer for a DBS protocol. If no protocol is available, it is highly recommended that they are developed in close collaboration with the manufacturer so that the protocols are distributed to all laboratories using the same platform.

HIV DNA

When correlating plasma and DBS samples HIV VL measurements, it is important to remember that DBS contains HIV infected cells that are not present in plasma. While DBS samples may never have the same limit of detection as plasma to detect HIV-1 RNA, the contribution of HIV proviral DNA and intracellular virus particles can lead to an overestimation of viral loads or positive DBS results when viraemia is undetectable in ART treated patients' plasma samples (305). Monleau *et al.* treated DBS samples with DNase and found that DNA contributes largely to the HIV viral load measurements in DBS samples (319). The BioMerieux NucliSens easyQ platform is based on isothermal RNA amplification so HIV DNA is not detected. It can be expected that the levels of proviral DNA and intracellular virions in a sample will differ between patients, and within a single patient due to biological variation. This would imply that a standard correction would be inaccurate and a more detailed procedure would be necessary. The potential confounding of DNA in low VL measurements needs to be researched further and correlated to clinical conditions of HIV positive patients. As DBS adds more variability (-0.77 to 0.65 $\log_{10}$/mL), it is essential to critically review the laboratory procedures for DBS and minimize variability of each sample process step.

Because of the difference in sample volume and the presence of HIV DNA as well as cellular HIV RNA in DBS samples, the quantitation of HIV is complex. In our opinion, a carefully designed and executed reference curve for DBS samples needs to be made for each assay and population. It is therefore recommendable to contact the manufacturer and discuss a DBS protocol that includes these variables. When manufacturers provide a DBS protocol, DBS testing can become standardised and easier to be used for laboratories starting to use DBS for HIV VL.

Conclusion

The results shown in this study are comparable with previously published reviews (55, 297). The review published in 2009 included in-house HIV VL assays while in this study, we focused on commercially available assays and assessed the quality of the papers. Although not all points on the STARD checklist are equally important, distribution of HIV VL or population description, are important.

When challenges surrounding the pre-extraction and analytical stages are resolved, DBS can be used as an alternative to plasma for HIV VL quantitation based on the suggested threshold of 5,000 c/ml.

HIV VL in infants is high with interquantile ranges of 0.5 million to almost 5 million copies/ ml at 6 weeks of age (320) in the absence of prophylactic antiretrovirals, suggesting that DBS are a suitable sample (321). As more mothers and infants receive antiretrovirals to prevent mother to child transmission of HIV, lower HIV RNA levels in infected babies will be observed. Assays for HIV VL and EID detect nucleic acids and are performed on similar laboratory equipment and HIV VL assays have been evaluated for early infant diagnosis. Using the same platform and consumables for two diagnostic tests, the economies of scope would have advantages for clinical settings. If HIV VL and EID testing was performed on a single platform, standardisation and a robust consensus for sampling, storing, and processing DBS samples would be essential to allow successful implementation of DBS samples.

The trade-off between accuracy, costs and accessibility of syphilis screening assays

Chapter 4

4 The trade-off between accuracy, costs and accessibility of syphilis screening assays

4.1 Introduction to publication

Chapters 4, 5 and 6 address objective 2 of the thesis, which is to evaluate the use of DBS to assure the quality of POCT in remote settings.

The WHO and Centre for Disease Control and Prevention (CDC) advocate the implementation of POCT with a quality assurance method in place (322). HIV and syphilis POCTs are currently used at antenatal care clinics (ANC) in Tanzania without rigorous QA methods in place.

The POCTs currently used in Tanzania have already been evaluated in various African countries (10, 22, 323-325). The study performed by Lyamuya *et al.* evaluated five HIV POCTs in Tanzania, and all were highly sensitive and specific (99.4 -100%) (324). The thesis did not reassess the performance of HIV POCTs but extended on the research performed by others.

Because of the limited number of studies evaluating syphilis POCT, we performed an evaluation comparing POCT to a laboratory based screening assay, using *Treponema pallidum* Particle Agglutination (TPPA) as reference method. Initially, the evaluation performed in this chapter comprised of four laboratory assays, to assess the most sensitive method. The four assays were; the TPPA, *Treponema pallidum* Haemagglutination Assay (TPHA), Enzyme Immuno Assay (EIA), and POCT. The initial idea was to compare the 4 methods, but the focus of the paper changed to discuss accessibility, costs and performance of the POCT and EIA. A version of the manuscript describing the evaluation of the four methods is described in Annex 10.4.

4.2 RESEARCH PAPER

The trade-off between accuracy, costs and accessibility of syphilis screening assays

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Status: Not yet submitted

Contributions: The candidate initiated the study, developed laboratory protocols, analysed the data, and drafted the manuscript. DM and RWP provided supervision throughout the study and made major contributions to editing the manuscript. JT was responsible for POCT data collection and revision of the manuscript. JC, AA, and JM provided supervision for laboratory work and handling of the data. BDC provided guidance on the data extraction from LMS and performed various data quality checks. BZ and MU planned sample collection and arranged it to use these samples in the laboratory. The candidate managed each round of suggestions and comments from co-authors.

The candidate **The supervisor**

Abstract

The availability of rapid and sensitive methods to diagnose syphilis facilitates screening of pregnant women, which is one of the most cost-effective health interventions available. We have evaluated two screening methods in Tanzania: an enzyme immunoassay (EIA), and a point-of-care test (POCT). We evaluated the performance of each test against the *Treponema pallidum* particle agglutination test (TPPA) as reference method, kit costs, and the accessibility of testing in a rural district of Tanzania. The POCT was performed in the clinic on whole blood, while the other assays were performed on plasma in the laboratory. Samples were also tested by the rapid plasma reagin (RPR) test.

With TPPA as reference assay, the sensitivity and specificity of EIA were 95.3% and 97.8%, and of the POCT were 59.6% and 99.4% respectively. The sensitivity of the POCT and EIA for active syphilis cases (TPPA positive and RPR titre ≥1/8) were 82% and 100% respectively. Only 15% of antenatal clinic attenders in this district attended a health facility with a laboratory capable of performing the EIA.

Although it is less sensitive than EIA, its greater accessibility, and the fact that treatment can be given on the same day, means that the use of POCT would result in a higher proportion of women with syphilis receiving treatment than with the EIA in this district of Tanzania.

Introduction

The prevalence of syphilis is high among pregnant women attending antenatal clinics in sub-Saharan Africa (326). Syphilis in pregnancy can have devastating effects on the developing foetus and is a major cause of stillbirths and neonatal deaths in Africa (327). Screening and treatment of pregnant women with a single dose of benzathine penicillin before the third trimester could prevent up to 305,000 stillbirths per year (3, 328).

Latent syphilis can only be diagnosed serologically. Laboratory based assays such as the *Treponema pallidum* particle agglutination assay (TPPA) and rapid plasma reagin (RPR) test are widely used. As the agglutination is interpreted by a technician, the test result is subjective. Enzyme immunoassays (EIA) are now recommended for syphilis screening in Europe (329). They are easy to use, provide objective results, and are well adapted to high throughput laboratories; but they are more expensive than the other assays, require equipment (a plate washer and a plate reader), and require cold storage of consumables, which is a limiting factor for some settings (330, 331).

These laboratory based assays are not available in rural health facilities in Africa. POCTs are easy to perform, require only a drop of blood collected by finger prick, and do not require refrigeration; they could enable same day testing and treatment for syphilis at any health facility but are less sensitive than laboratory based assays (332).

Selecting a screening assay, particularly in an African country, should not be solely based on the performance of the assay. Besides the performance of a test, cost, necessary equipment, cold chain requirements, and complexity of executing tests should be taken into consideration. Selecting a screening assay is therefore often a trade-off between the performance of the assays, cost, and accessibility for patients to be screened. Comparative evaluations that include these three aspects for syphilis screening have not been published previously (333). This study evaluates two screening assays, a POCT and an EIA, to review the trade-off between performance, cost, and accessibility of syphilis screening assays in an African district.

Methods

Samples and Field Procedures

The Kisesa open cohort is a well-established ongoing community-based study in Northern Tanzania (334). The cohort study uses regular demographic surveillance with serological surveys, providing data on HIV incidence and prevalence (335). Subjects that accepted voluntary counselling and testing (VCT) were tested for HIV and syphilis using POCT. All subjects with a positive syphilis result were given free medical treatment according to Tanzanian

government guidelines, and all those positive for HIV were referred to the Tanzanian care and treatment centre. The study was approved by the ethical review committee of the National Institute for Medical Research (NIMR) in Tanzania and the ethical committee of the London School of Hygiene and Tropical Medicine.

Whole blood was collected by venepuncture into heparinised tubes from consenting subjects, and transported to the NIMR laboratory in Mwanza. Within 24 hours the blood was centrifuged and stored at -20°C. Samples were collected from April 2010 until September 2010 and were tested until March 2011. Samples were bar-coded to ensure anonymous testing. Double data entry was used to enter the results. Results were entered automatically (EIA) or manually into the Laboratory Information Management System (LIMS).

Point-of-care test

The SD bioline syphilis 3.0 POC tests (Standard Diagnostics, Kyong gi-do, Korea) were performed by trained clinicians, with whole blood samples collected by finger prick. A timer was used to ensure that the test was read after exactly 15 minutes. The manufacturer's instructions were followed.

TPPA

A total of 2099 plasma aliquots were allowed to come to room temperature and tested by TPPA (Fujirebio, Tokyo, Japan) according to manufacturer's instructions. TPPA results were read by two trained and experienced technicians. The reading of the TPPA occurred while masked to results of other tests. Discordant results between the two technicians were discussed and one outcome was agreed by consensus. Results were deemed indeterminate for biologically reactive samples or when a conclusive outcome could not be obtained due to difficulty in interpretation or lack of technician's agreement.

Enzyme Immuno Assay

The Syphilis Enzyme Immuno Assay (EIA)(Lab21 healthcare, Kentford, UK) became available at the midpoint of the study and was performed on 1041 samples (49.6%). It was performed according to the manufacturer's instructions and read by Optical Density (OD) 450/620nm using an automated reader (DTX 800, Beckman Coulter, USA) which calculated the cut-off according to the instruction manual.

RPR

Quantitative RPR (BD Macro-vue RPR, Beckton Dickinson, Sparks MD, USA) was performed on all samples according to the manufacturer's instructions. Since adverse pregnancy outcomes due to syphilis are seen in women with RPR titres of ≥ 1:8 (327), an active syphilis infection was defined for the purpose of this study as TPPA positive and RPR titre ≥1/8 (19).

Sensitivities, specificities, positive predictive values and negative predictive values were calculated according to standard methods. The agreement between various methods was tabulated. Microsoft Excel (Microsoft Corp., USA) and the statistical package Stata 11 (Stata corp LP,Texas, USA) were used to analyse the results.

Access to screening

We visited all 51 health facilities in one district of Mwanza Region (Geita District), to ask how many women attend each antenatal clinic per month (336).

Results

Point- of- care test

The POCT was evaluated with a set of 2099 samples. Of these, 359 (17.1%) samples were positive by TPPA and 225 (10.7%) by POCT. With TPPA as reference, the POCT had 11 false positive and 145 false negative results, giving a sensitivity of 59.60% (95% CI: 54.5-64.6%) and specificity of 99.40% (95% CI: 98.9-99.7%) (Table 4.1). There was a 92.6% agreement between POCT and TPPA. The positive and negative predictive values were 95.1% and 92.3%, respectively.

Table 4.1: The TPPA and POCT performances are given with RPR results divided into titres lower (<1/8) or higher than 1/8 (≥1/8).

TPPA	POCT	RPR			Samples
		<1/8	≥1/8	Neg	
+	+	71	41	102	214
+	-	21	9	115	145
-	+	3	0	8	11
-	-	44	9	1676	1729
Total		139	59	1901	2099

+= positive, - = negative, Neg=negative

Of the 145 false negatives by POCT, 115 (79%) were RPR negative and 31 were RPR positive. Fifty out of 2099 samples tested by POCT had active syphilis (positive TPPA and RPR titre ≥ 1/8), of which 41 were detected by POCT, giving a sensitivity of 82% (95%CI: 69.2% -90.2%) and specificity of 100%.

EIA

The EIA was evaluated with 1041 samples that were used to evaluate the POCT as it became available in the second half of the study. Of these, 18.1% of samples were positive by TPPA and 19.1% by EIA.

With TPPA as reference, the EIA had 20 false positive and 9 false negative results, giving a sensitivity of 95.2% (95% CI: 91.1-97.46%) and specificity of 97.7% (95% CI: 96.4-98.5%) (Table 4.2). There was a 97.3% agreement between TPPA and EIA. The positive predictive and negative predictive values were 90.6% and 98.9%, respectively. The EIA showed a sensitivity and specificity of 100% to detect active syphilis cases (Table 4.2).

Table 4.2: The TPPA and EIA performances are given with RPR results divided into titres lower (<1/8) or higher than 1/8 (≥1/8).

TPPA	EIA	RPR			Samples
		<1/8	≥1/8	Neg	
+	+	53	26	100	179
+	-	0	0	9	9
-	+	3	0	17	20
-	-	30	4	799	833
Total		86	30	925	1041

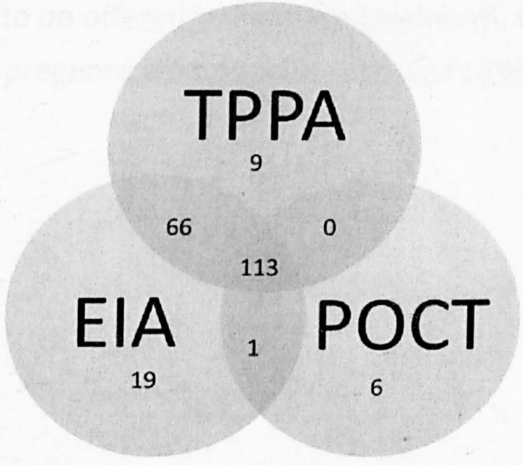
+= positive, - = negative, Neg=negative

All 29 samples for which a discordant result was obtained by the two laboratory methods were retested. On retesting, 8 out of 9 TPPA positive samples became negative and one became indeterminate, and 6 out of 20 TPPA negative samples became positive. Only four EIA positive samples changed outcome when retested (4 initially positive samples became negative out of 29 discordant results).

Comparison of EIA and POCT

Figure 4.1 shows the distribution of 214 positive out of 1041 samples identified by one or more of the screening assays.

Figure 4.1: Distribution of positive samples among the three assays.



The numbers represent positive samples detected by three assays (number is given in 3 circles), or by two assays (overlapped by two circles) or by one assay (given as number below the assay name). 214 positives detected by any of the three methods out of 1041 samples.

Costs of consumables

The kit cost (excluding labour) in Tanzania of one EIA reaction is $0.42- $0.93 and of one TPPA reaction is $0.46. The costs vary depending on the size of the purchased kits. This simple calculation does not include the number of negative and positive controls needed per run, which is higher for the EIA. The cost of the POCT was $ 1.10 per test in Tanzania.

Accessibility

Geita District has one district hospital, 8 health centres, which tend to be in larger villages, and 41 rural dispensaries. The EIA could only be performed at the district hospital, since health centres and dispensaries do not have centrifuges for plasma separation, cold storage capabilities, plate washers or readers. On average, 517 pregnant women attend the district hospital ANC for the first time per month, whereas 2849 attend a health centre or dispensary. If the prevalence of active syphilis is 2.3%, as found in this study, 77 pregnant women with active syphilis would be expected to attend an ANC in Geita District each month. Twelve of these would be attending the district hospital, and 65 would be attending health centres or dispensaries. With a sensitivity of 82%, the POCT would detect 63 of these, and enable them to be offered immediate treatment, whereas without the POCT only 12 of 77 pregnant women with syphilis (15%) would be identified.

Discussion

To our knowledge, this is the first study comparing POCT and EIA performed in an African setting. Additionally, this is the first study that includes costs, accessibility and diagnostic performances of syphilis screening assays into one evaluation.

It is important to note that this study was performed in Tanzania on Tanzanian samples, which potentially influences the results. African samples can contain high immunoglobulin levels due to other infections which potentially cause false positive test results (337, 338).

Although tests were performed and stored according to the manufacturer's recommendations, potential environmental effects by transporting and using the kits under tropical conditions could not be completely ruled out. Performing tests in an African country relies more heavily on the robustness of the tests than in developed countries. This could potentially influence the test results.

The reproducibility of the EIA evaluated in this study was good, with few samples giving discordant results on re-testing. Thesamples that were discordant had very low ODs, just above the cut-off. Therefore, EIA seems to be a more stable and reliable test compared to TPPA.

Adverse pregnancy outcomes due to syphilis are seen in women with RPR titres of ≥ 1:8 (327). In this study, the EIA had a higher sensitivity for the detection of these cases than the POCT (100% versus 82%), but a major limiting factor is that the EIA can only be performed when sufficient laboratory infrastructure is available. Most importantly, in Mwanza region, the POCT would enable more pregnant women with syphilis to be identified due to its greater accessibility, since pregnant women can be screened not only at the hospital but also at health centres and dispensaries. The RPR test is easier to perform than the EIA and requires less equipment, but requires access to a laboratory with electricity. It has been used to screen pregnant women in many developing countries, but the test results are difficult to interpret and results obtained in rural health facilities have not been encouraging (339).

In some countries blood is taken from pregnant women at rural health facilities and sent to a central laboratory to be tested for syphilis, but under these circumstances women with syphilis will only be treated if they return for their results (340). A study in Kenya found that less than 10% of pregnant women with syphilis received appropriate treatment when serological tests were performed at a central laboratory (341). When same day testing and treatment were made available in these clinics, more than 90% of infected women were treated (339).

A test which gives a result in 15 minutes, allowing patients to be tested and treated on the same day, may result in more cases being treated than a more sensitive laboratory test which requires patients to return for treatment at a later date The great advantage of the new POCTs for syphilis is that they do not require laboratory equipment or electricity, and can make same day testing and treatment for syphilis available at any health facility (336, 342). Based on the performance, costs and accessibility of both tests, the POCT is the best option for this and potentially many other settings in developing countries.

Conflict of interest

None to declare

Acknowledgements

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The evaluation of DBS as QA sample for syphilis POCT

Chapter 5

5 The evaluation of DBS as QA sample for syphilis POCT

5.1 Introduction to publication

The literature review identified a limited number of publications describing the use of DBS samples for syphilis serology (Chapter 2) (343, 344). Even though the procedure of using DBS samples has been described, the serological tests that were used are not commercially available any more. Additionally, test procedures were not rigorously developed, which suggested that a new evaluation would be necessary.

In order to use DBS samples as QA method for HIV and syphilis POCT, it is essential to establish the exact sensitivity and specificity of DBS compared to the gold standard sample, serum or plasma. This will allow a better interpretation of the concordance between POCT and DBS sample based test. And, as noted in chapter 1, evaluating an assay in the country where it will be used is essential to take biological and environmental factors into account.

This paper evaluates the use of three different syphilis serological assays, TPPA, TPHA and EIA with DBS samples, to identify the best performing assay for use with DBS compared to matching plasma samples. This paper also identifies one assay that is providing excellent results while the other two appear not to be suitable for DBS samples. Moreover, it also shows the importance of laboratory technicians being trained to obtain high sensitivities, particularly for assays that are subjective in result interpretation, as for haemagglutination assays as TPPA and TPHA.

5.2 Research paper

The development and validation of a novel method for quality assurance of syphilis serology using dried blood spots

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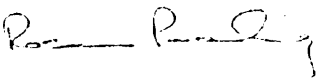
Contributions: The candidate initiated the study, developed laboratory protocols, performed laboratory tests, extracted and analysed the data, and drafted the manuscript. TV aided in developing protocols and testing samples. DM and RWP provided supervision throughout the study and made major contributions to editing the manuscript. JT was responsible for sample collection, sample process, and revision of the manuscript before submission. JM and JC provided supervision for laboratory work, handling and extraction of the data. BDC provided guidance on the data analysis and participated in the interpretation of results. AA, BZ and MU planned sample collection and contributed in writing of the manuscript. The candidate managed each round of suggestions and comments from co-authors. All authors read and approved the final draft prior to journal submission.

The candidate



The supervisor

Abstract

Background

Syphilis causes up to 305,000 stillbirths and neonatal deaths annually. These could be prevented if all pregnant women were screened, and those with syphilis treated with a single dose of penicillin before 28 weeks gestation. In recent years, rapid point-of-care tests have allowed greater access to syphilis screening, especially in rural or remote areas, but the lack of quality assurance of rapid testing has been a concern. We determined the feasibility of using dried blood spots as specimens for quality assurance of syphilis serological assays.

Methods

We developed DBS extraction protocols for use with *Treponema pallidum* particle agglutination assay (TPPA), *Treponema pallidum* haemagglutination assay (TPHA) and an enzyme immunoassay (EIA) and compared the results with those using matching plasma samples from the same patient.

Results

Since DBS samples showed poor performance with TPHA and EIA, only the DBS TPPA was used in the final evaluation. After retesting the discordant samples, DBS TPPA showed an adjusted sensitivity of 97.7% and a specificity of 99.4% compared to TPPA using plasma samples as a reference.

Conclusion

Based on the results, DBS samples can be recommended for use with TPPA for quality assurance of point-of-care syphilis testing.

Background

Syphilis causes up to 305,000 stillbirths and neonatal deaths annually (3). These could be prevented if all pregnant women were screened and treated with a single dose of benzathine penicillin before 28 weeks gestation (328). Syphilis testing occurs most commonly by laboratory based assays such as *Treponema pallidum* haemagglutination assay (TPHA), *Treponema pallidum*

particle agglutination assay (TPPA), rapid plasma reagin (RPR), or enzyme Immunoassay (EIA). These tests need to be used with serum or plasma samples and require a centrifuge, shaker, and refrigeration for the reagents. They are therefore less suitable than point-of-care tests (POCT) for use in rural or remote locations. POCT screening tests for syphilis that are sensitive and specific in detecting treponemal antibodies are now available (332). The main advantages of POCTs are that they are easy to use, can be stored at room temperature, and can be used with whole blood, collected with a finger prick. The Global Report on Preterm Birth and Stillbirth and modelling studies have identified syphilis POCTs testing and treatment as an urgent priority for reducing perinatal morbidity and mortality (345-347). Many countries have therefore started to scale up the use of POCTs in prenatal screening programmes for syphilis, but the lack of suitable methods for quality assurance (QA) is a serious concern.

Most QA methods have been developed to monitor the quality of tests performed in the laboratory, and are not designed for monitoring POCT usage by healthcare workers at remote locations (10). Dried Blood Spots (DBS) have been suggested to be a suitable QA methodology for HIV POCTs (39). DBS samples have been used in prevalence studies for syphilis serology, but without prior validation of the methodology (12, 13, 348). DBS samples have been evaluated with TPPA (344), TPHA (343), and an in-house EIA which is not commercially available (56). The TPHA used in the study by Backhouse *et al.* is no longer commercially available. The fourteen year old TPPA protocol used by Coates *et al.* did not include a control for biologically reactive samples, and the final testing concentration of DBS eluate was more diluted than with plasma, potentially leading to reduced sensitivity for samples with low antibody titres (344). The aim of this study was to determine whether DBS can be recommended for use as samples for QA of syphilis serological assays. Our objectives were to develop and validate DBS protocols for use with commercially available syphilis diagnostic assays, and to determine their performance in syphilis serological assays using plasma samples as a reference.

Methods

Research setting

The Kisesa open cohort is a well-established ongoing community-based study in northern Tanzania that conducts regular demographic surveillance on HIV prevalence and incidence (334). Subjects that opted for voluntary counselling and testing (VCT) were offered HIV and syphilis POCT (SD Bioline, USA) performed by trained and experienced technicians. If the VCT participant was treponemal antibody positive by POCT, free medical treatment was provided according to the Tanzanian government recommendations, and all those positive for HIV were referred to Tanzanian care- and treatment centres. The study was approved by the Medical Research Coordinating Committee of the National Institute for Medical Research in Tanzania (NIMR) and the ethical committee of the London School of Hygiene and Tropical Medicine.

Procedures

For the community based HIV study, DBS samples (Proteinsaver 903 filter paper, Whatman, GE healthcare, USA) were collected by finger prick, air dried at room temperature for at least 3 hours and stored with desiccants in individual ziplock bags at -20°C. After subjects donated a DBS sample, they were invited to opt in for VCT. Whole blood samples were collected from consenting subjects that opted in for VCT by trained clinicians and transported to the NIMR laboratory in Mwanza. Samples were bar-coded in the field to ensure anonymous testing. Within 24 hours, the blood samples were centrifuged and plasma was stored in 3 aliquots (1mL each) at -20°C. From all subjects participating in the serosurvey who opted for VCT, 1,645 samples were randomly selected for this study.

All syphilis serology assays were performed according to manufacturer's directions. The positive and negative controls supplied with the kits were used with every run. The technicians were blinded to other test results and TPHA and TPPA results were read by two trained laboratory technicians. All tests were performed in at the NIMR laboratory, which participates in the WHO external QA programs for TPPA and Rapid plasma Reagin (RPR) tests. An active syphilis case is defined as RPR positive, confirmed by a treponemal test. The

results were entered using the laboratory information management system.

The protocol development and evaluation was divided into two phases. In the first phase, we determined the feasibility of using DBS with TPHA, TPPA and EIA using 464 samples. During DBS protocol development, care was taken to ensure an equivalent DBS sample input was used, compared to plasma. In the second phase, the serological tests that work best with DBS were selected and validated with 1,181 samples. Plasma samples were used as the reference standard. The RPR test was performed on plasma samples that gave discrepant results between DBS and plasma samples to determine if any active syphilis cases might have been missed. Samples were prospectively collected from July through September 2010 and were tested until March 2011. During phase 1, the preliminary evaluation of different syphilis serological methods with DBS samples, TPPA, TPHA and EIA were all performed from the same DBS eluate as only one DBS spot was available. As a reference method, TPPA, TPHA and EIA were performed on matching plasma samples in parallel. To ensure blinded reading in the laboratory, plasma samples were tested before DBS samples. Additionally, the three tests on DBS were performed separately from each other, to maintain blindness to other test results.

TPPA

A total of 1,645 plasma aliquots were brought to room temperature and 25µl were used to test for treponemal antibodies by TPPA (Fujirebio, Tokyo, Japan), according to manufacturer's protocols, by laboratory technicians who routinely perform TPPA on plasma samples.

For DBS TPPA testing, the protocol was adjusted as follows. A 6mm disk was manually punched and eluted in 100µl Phosphate buffered Saline (PBS) with 0.05% Tween80 in a clean 96 flat wells plate, shaken for 2 minutes and eluted overnight at 4°C. Upon the next day, the plate was shaken 2 minutes and brought to room temperature. 25µl sample dilution buffer was added to the first column of a clean 96 U-shaped plate, 25µl DBS eluate was added and mixed thoroughly. 25µl of the mixture was transferred to a second column and 25µl sensitized particles were added to column one, 25µl unsensitized

particles to column two. Plates were covered and incubated for at least two hours at room temperature on a vibration free surface, before result interpretation. Discordant results between the two technicians were recorded as indeterminate. This protocol allows TPPA and HIV serological tests to be performed from one DBS spot.

TPHA

Plasma aliquots were brought to room temperature and 25 µl were used to test for treponemal antibodies by TPHA (Lab21 syphilis TPHA, Lab21 healthcare, Kentford, UK). TPHA plasma tests were performed according to manufacturer's protocols by trained laboratory technicians.

For DBS TPHA testing, the following protocol was developed; 25 µl of DBS eluate (obtained as described above) was added to 25µl sample diluent, mixed and divided over two wells (25µl each). 75µl test or control cells were added and incubated for 1 hour. For both TPHA and TPPA protocols, the final DBS sample elution volume was kept comparable with plasma sample volume. Discordant results between the two technicians were recorded as indeterminate.

EIA

Plasma aliquots were brought to room temperature and 50 µl were used to test for treponemal antibodies by Enzyme Immuno Assay (EIA) (Lab21 Syphilis Total Antibody EIA, Lab21 healthcare, Kentford, UK). EIA plasma tests were performed according to manufacturer's protocols by trained laboratory technicians.

A DBS EIA protocol was developed in collaboration with the developer of the assay. 40µl DBS eluate was added and incubated for two hours at 37°C. Plates were washed five times using an automated washer, 50µl conjugate was added, shaken and incubated for 30 minutes at 37°C, washed five times, 50µl substrate was added and incubated for 30 minutes at room temperature while kept in the dark. 50 µl stop solution was added and the wells were read as Optical Density (OD) 450/620nm using an automated reader (DTX

800, Beckman Coulter, USA) with cut-off limits calculated according to the instruction manual. The results were then entered directly into the laboratory information management system.

RPR

Quantitative RPR (BD Macro-vue RPR, Beckton Dickinson, Sparks MD, USA) was performed according to manufacturer's protocols using plasma samples by trained laboratory technicians.

Data analysis

The sensitivity, specificity, positive predictive value and negative predictive value were calculated according to standard methods. The agreement between various methods was tabulated. Microsoft Excel (Microsoft, USA) and the statistical software Stata 11 (StataCorp LP, Texas, USA) were used for analysis of the results. Newcombe-Wilson score methods were used to provide confidence intervals for sensitivity and specificity.

Results

Phase 1: Preliminary evaluation of different syphilis serological methods with DBS samples

During the first phase of the project, protocols for DBS samples were developed for TPPA, (DBS TPPA) TPHA (DBS TPHA) and EIA (DBS EIA). 464 DBS samples were tested with TPPA, TPHA, and EIA. Table 5.1 shows the sensitivity and specificity of all three syphilis serology tests using DBS samples compared to plasma samples. The DBS EIA was discontinued before the end of phase 1 because of the many false positive results (specificity 50.4%). The TPHA was also excluded from further testing because of low sensitivity (50.6%).

Table 5.1: Preliminary evaluation: The performance of three syphilis serological assays using Dried Blood Spots compared to plasma.

Plasma as reference (using the same assay)	Positive samples detected	Negative samples detected	Sensitivity (95% CI)*	Specificity (95% CI)
DBS TPPA† (n=463)	82/96	363/368	85.42% (77.0 – 91.1%)	98.91% (97.2-99.6%)
DBS EIA (n=282)	53/56	114/226	94.60% (85.4-98.2%)	50.40% (44.0-57.0%)
DBS TPHA‡ (n=445)	46/91	353/354	50.60% (40.5-60.6%)	99.20% (98.4-100%)

n= samples
* 95% confidence interval, † 1 TPPA DBS indeterminate excluded,‡ 19 TPHA indeterminate results excluded

To improve sensitivity of DBS TPPA, technicians were trained to use a lower cut-off for interpretation of the DBS TPPA test results, based on the agglutination patterns seen in phase 1. Reading was adjusted by altering the agglutination positive and negative thresholds applied for DBS samples because of the higher background compared to plasma samples.

Phase 2: Evaluation of DBS samples in comparison to matching plasma samples.

For the final evaluation of DBS TPPA, 1,181 matching DBS and plasma samples were tested, of which 179 plasma samples tested positive for treponemal antibodies, resulting in a 15.2% syphilis seroprevalence (Table 5.2)

Table 5.2: Correlation between detection of Treponema pallidum antibodies by plasma TPPA and DBS TPPA

(n=1147)		TPPA plasma		Total
		Positive	Negative	
DBS TPPA	Positive	169	10	179
	Negative	8	960	968
	Total	177	970	1147*

Sensitivity of DBS against plasma 95.5% (95% CI: 91.3 -97.7%)
Specificity of DBS against plasma 99.0% (95% CI: 98.1-99.4%)
* excluding 34 indeterminate results.

Of these 177 plasma positive samples, 66 were RPR positive (Table 5.3). Excluding 34 indeterminate results, DBS TPPA showed a sensitivity of 95.5% (95% CI: 91.3 -97.7%) and a specificity of 99.0% (95% CI: 98.1-99.4%) compared to TPPA plasma as a reference method.

Table 5.3: RPR titres of TPPA DBS positive samples

RPR titre	N=179
negative	113
1/1	13
1/2	19
1/4	13
1/8	7
1/16	4
1/32	6
1/64	1
>1/128	3

The optimisation resulted in a 10.1% increase in sensitivity. An overall agreement of 98.7% between the two readers was found. No non-specific reactivity (positive with unsensitized particles) with DBS samples was detected. Of the 34 indeterminate results for DBS TPPA, 30 (88.3%) were negative and 4 (11.7%) were positive for TPPA using plasma samples. Unfortunately it was not possible to retest the 10 false positive DBS samples since insufficient sample eluate was available by the time plasma and DBS results were compared. The eight samples that were false negative were likely either borderline samples or due to incorrect DBS TPPA test reading. The eight false negative matching plasma samples were retested with quantitative TPPA and quantitative RPR, as shown in Table 5.4.

Table 5.4: RPR results on the 8 Plasma samples with false negative DBS TPPA results.

Sample	DBS	TPPA*	RPR†
BBI99P	N	N	N
BBI792	N	N	N
BBI6GG	N	N	N
BBI5YI	N	N	N
BBI8ZC	N	1/80	N
BBI6LP	N	1/160	N
BBI6K6	N	1/320	N
BBI6NZ	N	>1/640	1/64

N= negative
* Quantitative TPPA
† Quantitative RPR

Four plasma samples were negative when retested with TPPA, suggesting a borderline sample or false positive reading when initially tested. Taking these four false positive plasma TPPA samples into consideration DBS TPPA sensitivity and specificity increased to 97.7% and 99.4%.respectively.

Discussion

We developed and validated protocols for the use of DBS samples as specimens for QA of syphilis serological assays. Unfortunately, we were not able to obtain acceptable performance for the use of DBS samples with the Syphilis Total Antibody EIA and Lab21 Syphilis TPHA. The EIA false positive results were primarily caused by a high background, possibly due to substances eluted from the filter paper and whole blood that adhered non-specifically to the wells. DBS samples tested with TPPA gave a sensitivity of 97.7% and specificity of 99.4% compared to plasma samples.

Of the eight samples that were false negative by DBS TPPA, four were negative when retested with quantitative TPPA on plasma samples and four were false negatives, of which two had relatively high TPPA titres (1/320 and 1/640). We tested all plasma samples using the RPR assay to determine if any of the false negative samples were from women with active syphilis, defined as being RPR and TPPA positive. Since only one false negative DBS sample was positive for RPR, DBS samples showed excellent sensitivity and specificity when used with TPPA and are potentially useful as samples for the diagnosis of syphilis and for QA.

Because TPPA is an agglutination assay, experience in reading results is essential and therefore training is necessary. 34 DBS samples (3%) were marked as indeterminate due to difficulty in interpreting the results or because of discordant reading by two technicians. Appropriate procedures for indeterminate samples have not been evaluated but retesting DBS eluates in duplicate seemed suitable to establish a final reading (data not shown). DBS samples can potentially be used for quantitation with TPPA, although it would require evaluation against titers obtained with plasma samples. Because of the subjectivity, it is recommended that TPPA should be read by two readers.

DBS TPPA has been used as a surveillance tool in a few studies (13, 344, 348) that used a protocol developed by Coates *et al.* (344). The DBS TPPA protocol developed in this study is an improvement to the protocol developed by Coates *et al.* as we adjusted the elution so that the sample input into the TPPA assays from DBS and plasma are comparable. We also included unsensitized particles in the procedure for control of biologically reactive samples.

Conclusions

As prenatal screening for syphilis using POCTs become widely implemented, a QA method appropriate for use with blood collected by a finger prick must be developed to assure the proficiency of POC testing in rural or remote areas. The aim of this study was to determine whether DBS can be recommended for use as samples for QA of syphilis serological assays. Based on the high sensitivity and specificity of DBS TPPA compared to Plasma TPPA, DBS can be recommended for use with TPPA, but not with TPHA or EIA, as a confirmatory assay for syphilis or as samples for QA of POCTs. Our study also showed the importance of training laboratory technicians in performing and reading the DBS TPPA, even when they are already trained in plasma TPPA. We obtained a 10.1% increase in sensitivity when technicians were more experienced in interpreting DBS TPPA agglutinations.

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The implementation of DBS as QA method

Chapter 6

6 The implementation of DBS as QA method

6.1 Introduction to publication

This Chapter extends on Chapter 4; the evaluation of syphilis POCT in comparison to a laboratory based screening assay (EIA) in Tanzania, and on Chapter 5; the development and evaluation of a laboratory protocol for syphilis serology on DBS samples. While this thesis assessed the performance of POCT and DBS for the detection of syphilis antibodies, it did not assess the performance of HIV POCT or HIV serology with DBS. The use of DBS samples for HIV serology has been evaluated in great detail and showed to be very sensitive (Chapter 2) (39, 52, 295, 349-352).

In this chapter, the use of DBS as QA sample is implemented to review the implementation process over a period of nine months, in ten ANC clinics in Tanzania. Additionally, the performance of the QA method to detect under-performing clinics is compared to syphilis proficiency panels, to review the performance in greater detail. The POCT testing performance of each clinic was obtained and compared to review potential differences in quality of testing.

By performing this study and making the protocols developed for this study available online, the method could potentially be used and scaled up as QA method for POCT at regional or even country level.

6.2 RESEARCH PAPER

The implementation of a quality assurance method for Point- of- Care Tests for HIV and Syphilis in Tanzania

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Contributions: The candidate initiated, implemented the study and drafted the manuscript. TV aided in handling the samples and aided in the interviews. DM and RWP provided supervision throughout the study and made major contributions to editing the manuscript. JM and JC were responsible for sample collection, and sample process. JT provided guidance on the data analysis and participated in the interpretation of results. HK planned and supervised testing of the samples and collaborated in writing of the manuscript. The candidate managed each round of suggestions and comments from co-authors. All authors read and approved the final draft prior to journal submission.

The candidate **The supervisor**

Abstract

Background

Quality assurance (QA) programmes, which are routinely used in laboratories, have not been widely implemented for point-of- care tests (POCTs). A study was performed in ten health centres in Tanzania, to implement the use of dried blood spots (DBS) as a QA method for HIV and syphilis (POCTs).

Method

DBS samples were collected for retesting at a reference laboratory and the results compared to the POCT results obtained at the clinic. In total, 2341 DBS samples were collected from 10 rural health facilities over a period of nine months, of which 92.5% were correctly collected and spotted.

Results

The QA method was easily implemented by healthcare workers under routine conditions in Northern Tanzania. For HIV, 967 out of 972 samples (99.5%) were concordant between DBS and POCT results. For syphilis, the concordance of positive syphilis tests varied between clinics, ranging from 38% - 89% with a mean of 64% (median 70%). The concordance of negative samples between syphilis POCT and laboratory based test using DBS varied from 93% to 99%, with a mean of 97%.

Conclusion

Overall, the quality of testing varied significantly at clinics and QA results can be used to identify clinics where healthcare workers require remedial training, suggesting the necessity for stringent quality assurance programmes for POC testing. As Tanzania embarks on scaling up HIV and syphilis testing, DBS can be a useful and robust tool to monitor the quality of testing performed by healthcare workers and trigger corrective action to ensure accuracy of test results.

Background

Point-of-care diagnostic tests (POCTs) are increasingly used in both developing and developed countries (353). They allow same day testing and treatment at remote locations where no laboratory support is available. Quality control measures, which are routinely used in laboratories, have not been widely implemented for POCTs. The World Health Organization and US Centre for Disease Control and Prevention advocates the implementation of POCT with a quality assurance method in place (322).

In Tanzania, the Ministry of Health currently recommends the use of POCTs to screen pregnant women for HIV and syphilis, but a quality assurance (QA) method has not been implemented (336). Dried blood spots (DBS) have been suggested as a potential method for QA for HIV POCT (39), but this has not been implemented under routine testing services.

In this study, we implemented DBS as a QA method for both HIV and syphilis POCT in Tanzania. The study aims were; (1) to evaluate the feasibility of using DBS as a continuous QA method for end users in a rural setting of a developing country; (2) To review the performance of the various clinics involved in syphilis and HIV testing; (3) to compare this QA method with external QA using proficiency panels, and (4) to make the project's protocols available online.

Methods

Setting

As part of a larger study that implemented syphilis POCT in seven developing countries, the Geita District in Northern Tanzania was chosen for this study (336). Ten out of 51 ANC clinics that participated in the main study were selected based on location, type of clinic, and willingness to participate. The study was conducted in ten primary health clinics that offered antenatal care (ANC) and HIV voluntary counselling and testing (VCT) services. To represent the various types of Tanzanian clinics providing ANC and VCT services, the district referral hospital, four health centres and five dispensaries were included in this study. The classification of clinic type is primarily based on

the number of personnel and which services are offered to the patients. The study population were pregnant women and their partners. The study was conducted over a period of nine months, from January till September 2011.

Routine services offered

The QA method was incorporated in the national routine ANC and VCT services given at ANC clinics in Tanzania. Patients accepting HIV and syphilis testing were tested on-site using the SD Bioline syphilis 3.0 (Standard Diagnostics, Kyong gi-do, Korea) on whole blood obtained by finger-prick. The national HIV POCT testing algorithm consisted of SD Bioline HIV 3.0 (Standard Diagnostics, Kyong gi-do, Korea), Determine HIV- 1/ HIV-2 (Abbott Laboratories, Wiesbaden-Delkenheim, Germany) with Unigold (Trinity Biotech, Dublin, Ireland) as the tie-breaker test. All tests used whole blood obtained by finger prick and were performed according to the manufacturer's recommendations. Free medical treatment was provided to all patients testing positive for syphilis. HIV positive patients were referred to Tanzanian care- and treatment centres. All services were performed according to Tanzanian guidelines.

Implementation of QA method in routine practise

The patients were requested to donate blood for quality control purposes and asked to provide oral consent by the health care worker (HCW). Ethical approval was obtained from ethical committees at the London School of Hygiene and Tropical Medicine and at the National Institute of Medical Research, NIMR, Tanzania. If consent was given by the patient, whole blood was obtained by finger prick according to standard protocols (322). Whole blood was applied to the POCT and the DBS was spotted afterwards. The HCW was instructed to not perform an additional finger-prick when the DBS could not be completely filled, to minimize discomfort for the patient. Only the POCT outcome, date of testing, patient number and the clinic name were recorded on the study form.

At the start of the study, HCW that performed HIV and syphilis testing received a one- day centralised training for the collection of DBS samples. During the training, HCW learned about the method and received hands-on training of spotting blood onto filter paper, labelling DBS samples, drying, and storing DBS samples. After the training, HCW were given filter papers (Protein Savercard 903, Whatman, GE healthcare, Wisconsin, USA), desiccant bags (indicating desiccants, Brownell ltd, UK), a dry rack (GE healthcare, Wisconsin, USA) and ziplock bags (Minigrip, Netherlands) to store the DBS samples. Syphilis POCT kits (SD Bioline syphilis 3.0,Standard Diagnostics, Kyong gi-do, Korea) were purchased and transported to the hospital, from where the kits were distributed to the clinics. To minimise the workload, DBS samples were stored up to 10- 15 cards per ziplock bag with desiccants at the clinic. Risk of cross contamination for serological testing was limited due to the tucked cover of the DBS cards protecting the blood spots.

To minimise the workload of the busy HCW, identification stickers were used. One sticker was placed on the DBS and the other on a registration form where the matching POCT results could be encircled. Laboratory technicians were masked to the POCT results and the ANC registration forms containing the POCT results were entered into Epi-info v. 3.5.3 database (Centre for Disease Control and Prevention, Atlanta, USA). Interviews were conducted to obtain feedback of the HCW in June 2011. All HCW found the method easy and relatively quick to use. According to the HCW, the QA process time including spotting, labelling and recording result varied from 2-6 minutes. As most HCW provided counselling during the waiting time before the POCT results could be read, the DBS QA method did not cause any delay as this was done during the 15 minute waiting time.

To review the feasibility of the QA methodology for large scale application, laboratory time was monitored. The time estimates in Table 6.1 are based on an experienced laboratory technician and do not take preparation of materials (e.g. reconstitution of buffers) into consideration.

Table 6.1: Sample processed and data entered per hour per one technician, divided per activity.

Activity	Samples processed per hour	Data entry required?	Samples entered per hour
Register quality of DBS	200	yes	225
Punch and prepare masterplates	180	no	-
Murex HIV test	135	automated	-
TPPA syphilis test	500	yes	200
Vironostika HIV test	190	automated	-
Clinic record form entry	NA	yes	140
Average	241		188

Shipment and laboratory methodology

The ANC registration forms and DBS samples were collected in March, June and October. Samples were counted and checked for humidity (changed desiccants if required) before shipment at ambient temperature to the laboratory. On average, the time between sampling and arrival at the laboratory where testing took place was 95 days (minimum= 62 days, maximum= 142 days). The DBS samples were transported at ambient temperature to the Leiden Cytology and Pathology Laboratory, Leiden, The Netherlands where the samples were tested. Upon arrival at the laboratory, the quality of DBS samples were reviewed and recorded according to the online protocol (354). The DBS quality data was entered into epi-info. The DBS were placed with new desiccants in a ziplock bag and stored at 4°C until tested. To allow fast interpretation of the results, an excel file was developed that imports Epi-info data. The Excel file automatically presents data from each clinic graphically which could aid supervisors to adequately identify and provide detailed feedback to clinics requiring supervision when the QA method is implemented. All documents are freely available online (354).

Syphilis testing

Treponema pallidum Particle Agglutination assay (TPPA, Serodia,Tokio, Japan) was performed as described previously (Smit *et al* submitted). Briefly, 6mm spots were punched with a DBS puncher (PerkinElmer, Greenville, SC, USA) in a 96 flat well plate. 100µl Phosphate buffer Saline (PBS) with 0.05%

tween20 was added, shaken for 2 minutes and eluted overnight at 4°C. DBS eluates were shaken for 2 min and 25µl was transferred to a clean 96 U well plate. 25µl specimen diluent was added, mixed thoroughly and 25µl was transferred to a second column. 25µl sensitized or unsensitized particles were added and agglutination was read after 2 hours incubation. Results were read independently by two technicians.

HIV testing

An Enzyme Immunoassay (EIA,) Vironostika HIV EIA (BioMerieux, France), was used to review the HIV POCT results. The EIA was performed as described by the Center for Disease Control and Prevention (CDC) (Vironostika protocol) (355).

Proficiency panel results

For quality assurance purposes, various measures were taken at clinic level. With every new syphilis POCT lot, a known positive and negative was tested at the clinic. Additionally, syphilis proficiency panels were made and regularly brought to clinics to assess the HCW performance. The syphilis proficiency panels consisted of four dried tubes samples (DTS) as described previously (356). Briefly, DTS samples were made in the laboratory by drying aliquots of known positive and negative sera in small tubes and panels were sent to the clinics. The proficiency panels were sent to the clinics from July 2010 till January 2011 every month. The proficiency panels consisted of four dried tube specimens with known serostatus and changing number of positive and negative samples. At the clinic, HCWs reconstituted the DTS with PBS-tween20 buffer and the reconstituted serum was used to test with syphilis POCT. Results were recorded and sent back to the laboratory.

Statistical analysis

Double data entry into Epi-info 3.5.3 (CDC, Atlanta, USA) was applied and data was analysed in Stata12 (Stata Corp, Texas, USA). Data was analysed using McNemar Chi^2 to assess agreement of results from different tests. POCT results were categorised into 4 groups and coded 1-4; true positive, true

negative, false positive, and false negative, to compare the false negative and false positive results separately to the correctly concordant results. Multinomial logistic regression was used to obtain relative risk ratios (RR) and adjusted for clinic, month, quality of DBS samples, and HIV test result to obtain adjusted relative risk ratios (ARR). Likelihood ratio tests (LRT) and 95% confidence intervals (95% CI) were used to assess the significance of the association.

Results

DBS as QA method

To evaluate the implementation of the QA method across the ten antenatal care clinics (ANC), the quality of DBS samples was reviewed. In total, 2,341 samples were collected. Of these samples, 140 samples (6.07%) were excluded due to invalid syphilis POCT or TPPA test result (e.g. TPPA indeterminate or no POCT result) (124 samples), missing forms (10 samples), or the DBS sample was unusable (6 samples), either because of fungal growth or because it did not contain enough blood to obtain at least one 6mm punch. Of the remaining 2,201 cards, containing 11,005 spots, 10,179 (92.5%) were correctly spotted. Of the 7.5% incorrect spots, 356 spots (3.2%) were empty.

The quality of testing at Tanzanian clinics

HIV and syphilis tests are routinely performed as part of the ANC services provided to pregnant women visiting these clinics. Due to a prolonged stock-out of HIV POCT during the study period, many women (55.8%) were not screened for HIV while still receiving a syphilis test (Table 6.2).

Table 6.2: Samples collected per clinic and proficiency panel results per clinic.

		POCT performed per clinic		Proficiency panel results	
Clinic	Clinic type	Number of HIV tests	Number of syphilis tests	last 3 months*	overall (7months)
1	District referral hospital	272	422	100%	86%
2	Dispensary	13	109	100%	89%
3	Dispensary	45	71	100%	86%
4	Dispensary	72	172	100%	88%
5	Dispensary	15	113	100%	90%
6	Dispensary	24	150	100%	93%
7	Health Centre	84	337	100%	79%
8	Health Centre	257	258	100%	92%
9	Health Centre	25	283	100%	64%
10	Health Centre	165	286	100%	95%
	Total	972	2201	100%	86%

*last 3 months of proficiency panel results (November - January 2011)

Out of the 2201 samples included in this study, 972 (44.2%) had recorded HIV test results. In total, 43 patients (4.4%) were identified as positive by SD Bioline HIV POCT of which 30 were retested by Determine HIV POCT as part of the national testing algorithm. All Determine tests confirmed HIV positivity. It is unclear whether the 13 positives not confirmed by a second POCT (Determine) were identified as HIV positive or negative and they were therefore excluded from further analysis. Of the 30 positive samples, 28 (93%) were positive by DBS tests, 1 was negative and 1 sample could not be tested due to insufficient material for confirmatory testing. 929 (99.7%) samples were HIV POCT negative of which 3 (0.3%) were positive by DBS tests.

Out of 2201 DBS samples collected, 130 (5.9%) were positive by syphilis POCT and 202 (9.2%) were positive by DBS TPPA. In total, 2012 samples were negative by syphilis POCT and 1999 were negative for DBS TPPA. The concordance of positive syphilis tests varied between clinics, ranging from 38% - 89% with an average of 64% (median 70%) (Figure 6.1). The concordance of negative samples between both methods varied from 93% to 99%, with an average of 97% (Figure 6.1 and Figure 6.2). Two clinics (clinic 2 and 7) performed significantly different from the other clinics in the concordance of syphilis results ($P< 0.05$); clinic 2 performed significantly better and clinic 7 significantly worse than the other clinics (Figure 6.1). Compared to the district referral hospital, patients visiting clinic 2 had a Relative Risk of 3.7 for a false positive result (AOR=3.7, $p <0.05$).

Figure 6.1: Percentage of positive concordance of syphilis POCT per clinic

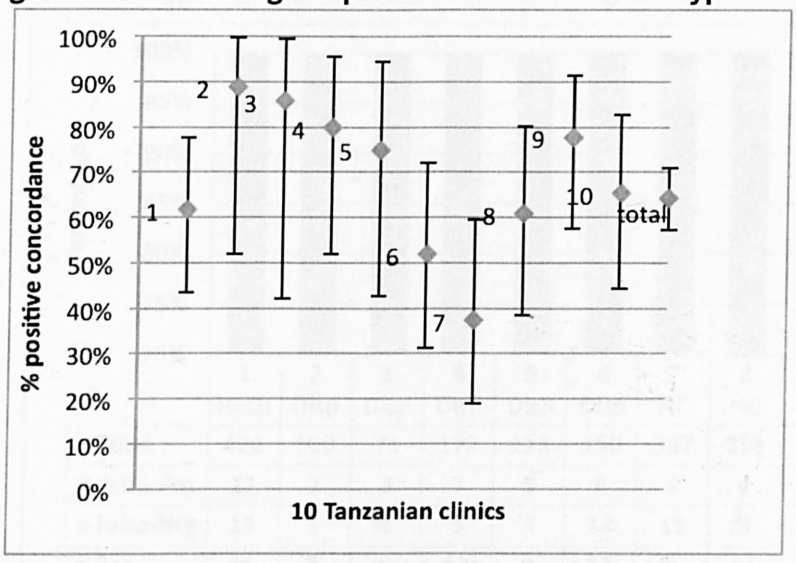
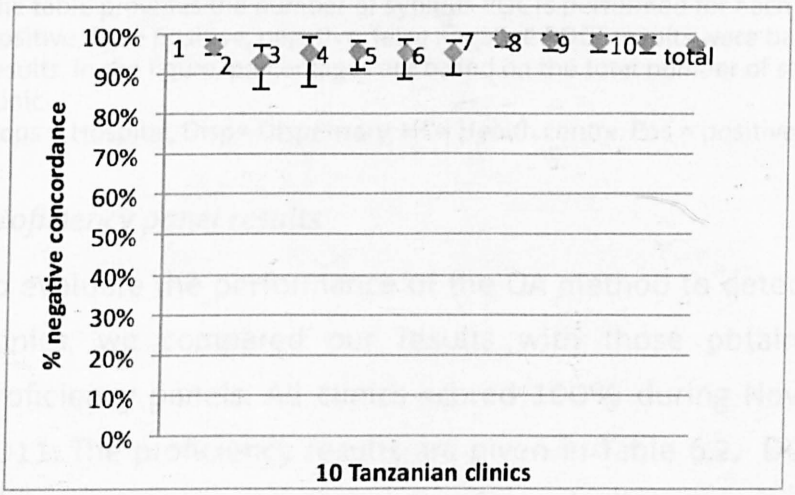
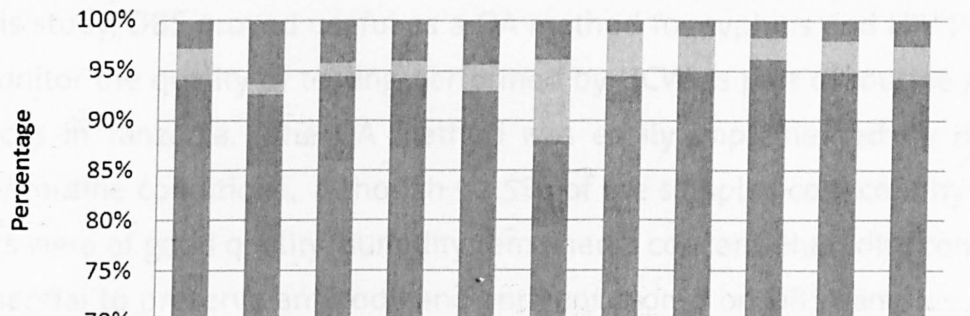


Figure 6.2: Percentage of negative concordance of syphilis POCT per clinic



No remedial training for POCT testing or DBS collection was given during the study period. To evaluate the performance of the clinics over time, January and August, the months when the largest number of samples was collected, were compared. There was no statistically significant difference in concordance of POCT and laboratory tests (ARR=0.557, $p > 0.05$) between the two months. This finding suggests that the clinics did not perform differently at the beginning or at the end of the study. The quality of DBS cards had no effect on the proportion of discordant POCT and DBS TPPA results (likelihood chi^2 ratio = 0.346, P= 0.556).

Figure 6.3: Syphilis POCT results evaluated against DBS as reference method



	1 Hosp	2 Disp	3 Disp	4 Disp	5 Disp	6 Disp	7 HC	8 HC	9 HC	10 HC	Total
Total	422	109	71	172	113	150	337	258	283	286	2201
False-Pos	12	7	3	7	5	6	4	4	5	6	59
False-Neg	13	1	1	3	3	12	15	9	6	9	72
Pos	21	8	6	12	9	13	9	14	21	17	130
Neg	376	93	61	150	96	119	309	231	251	254	1940

The table provides the number of syphilis POCTs performed for each clinic. The 4 categories; positive, false positive, negative, false negative POCT results were based on DBS TPPA results. In the figure, percentages are based on the total number of samples collected per clinic.
Hops = Hospital, Disp= Dispensary, HC= Health centre. Pos = positive, Neg= Negative

Proficiency panel results

To evaluate the performance of the QA method to detect underperforming clinics, we compared our results with those obtained using syphilis proficiency panels. All clinics scored 100% during November till January 2011. The proficiency results are given in Table 6.2. During the 7 months when proficiency panels were performed, clinic 9 scored the poorest with an average of 64% correct, while obtaining good performance in our QA. Clinic 7 scored 79% correct in the proficiency panels while having a poor performance by our QA. The proficiency panel results did not correlate well with the results found by our QA method.

Discussion

In this study, DBS proved useful as a QA method for syphilis and HIV POCT to monitor the quality of testing performed by HCW as part of routine ANC services in Tanzania. The QA method was easily implemented by HCW under routine conditions. Although 92.5% of the samples collected by the clinics were of good quality, humidity remained a concern. Humidity control is essential to preserve antibody and antigens stored on DBS samples and to prevent growth of mould. Storage of desiccants at the clinics for several months proved difficult as they became humid, especially during the rainy season. More suitable containers with an airtight lid should be used. Additionally, long term storage (e.g. more than 4 weeks) at clinics should be prevented as this potentially could lead to loss of samples or increase risks of unfavourable storage conditions that could lead to degradation of antibodies or antigen (23).

This study showed the possibility to seamlessly integrate HIV and syphilis POCT quality assurance, making it possible to have one QA method for preventing mother-to-child transmission of HIV services.

The DBS method has advantages over proficiency panels as this method gives insight into the quality of testing during routine patient services. Proficiency panel samples are tested by HCW with the knowledge that they are being monitored and only contain four samples. As only one proficiency panel is sent out to every clinic, only one HCW is evaluated while with DBS QA, all HCWs are included in the QA procedure. These reasons could potentially affect the proficiency panel result and could cause misinterpretation of the actual testing quality during routine patient services.

The overall performance of the 10 clinics for syphilis POCT was lower than the manufacturer's claim, although in agreement with earlier evaluations in Tanzania that found a sensitivity of 59.6% when using whole blood, compared to TPPA using plasma (332) (chapter 5). The low POCT performance should be interpreted with caution as differentiation cannot be made between discordant test result and incorrect interpretation or recording of the results by HCW. Worker turnover may be one of the reasons for low perfor-

mance as the replacement worker may not have been properly trained. The strength of this study is that the QA method can be evaluated continuously under routine clinical conditions. Because of this, the sample quality can be influenced by, for example, environmental influences during incorrect storage at the clinic or inter and national transportation of the kits. Based on our results, the quality of testing varied significantly between the ten clinics, even though all clinics used the same syphilis POCT lots. The hospital and dispensaries performed better than the health centres when using DBS as reference method. These QA results can be used to identify clinics where remedial training in the performance of syphilis POCTs is required.

Unfortunately, it was not feasible to monitor the costs of this QA method. Although the process time in the laboratory was relatively quick, this QA method can be more costly than, for example, the use of proficiency panels. By combining this QA for multiple infectious diseases as we did for HIV and syphilis, the economies of scope would limit the QA costs compared to setting up two independent QA methods.

To use this QA method in practice, the number of samples that should be collected per clinic depends on the setting. The POCT performance (e.g. sensitivity and specificity), incidence and prevalence of the infectious disease, robustness of the test outcome, staff turnover, as well as past QA results need to be taken into account in determining the number of samples that are required to obtain good insight in quality of POCT at the clinics (357).

Conclusion

To the authors' knowledge, this is the first paper describing the implementation of DBS samples as QA method for HIV and syphilis POCT. The key findings of our work suggest the necessity for stringent quality control of POCT as the quality of testing varied significantly between clinics. On average, the concordance of positive samples was low. The DBS QA method potentially provides a better solution to assure the quality of POCT testing, in comparison to proficiency panels in Africa. The QA method evaluated in this study could be easily rolled out in developing countries to improve and assure the quality of POCT testing.

Competing interest

The authors declare that they have no competing interests

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Development of a surveillance method for the causes of fever in children

Chapter 7

7 Development of a surveillance method for the causes of fever in children

As Chapters 4, 5 and 6 answered objective 2, this Chapter aims to provide an answer to objective 3. The potential use of DBS to improve syndromic management by surveillance of infectious diseases is assessed in the context of febrile children.

7.1 Introduction

Fever is one of the most common presenting symptoms in children. Fever in children can be classified into three broad categories, although overlap does exist. The first group is acute fever with an obvious focus, such as: respiratory infections, childhood exanthems, septic arthritis, meningitis, hepatitis, encephalitis, and enteric infections. The second, more complex, category is acute fever with nonspecific localizing signs. The last category is chronic fever with no clear pathogenic cause or diagnosis, despite adequate investigation.

Distinguishing between benign and serious causes of fever in young children is worrisome for health care workers all over the world. The differentiation between viral infection and "occult bacteremia" (clinically significant presence of bacteria in the blood stream) is the most important distinction that mostly relies on laboratory tests. The great range of possible causes and frequent lack of laboratory tests forces health care workers in developing countries to start treatment without a clear clinico-pathological foundation. This project focuses on acute fever without localised signs since this is a problem affecting small children under ten years of age and very heavily depends on laboratory diagnostics to determine the causative agent.

To analyse the causes of fever outside urban areas where it is not possible to collect and analyse blood samples, different approaches are needed that do not demand traditional whole blood collection and analysis.

The need for an easily collected sample method in combination with the ability to detect life threatening pathogens could provide the essential information to determine the prevalence, and seasonal characteristics of the

specific causes of fever and associated deaths. This method could provide information for evidence based policy and treatment guidelines.

To develop a surveillance method close to the given characteristics described above, the aim was to investigate the diagnostic accuracy of DBS for the collection of whole blood. Real-Time PCR was selected as detection method because it is fast, allows detection of multiple targets in one reaction (e.g. multiplex), is sensitive, specific, and can be performed on any Real-Time PCR machine with minimal adjustments. To obtain the highest sensitivity, priority was given to validate monoplex PCRs at this stage of the proof-of-concept study, before combining PCR assays into one reaction. By performing assays independently, every assay can be optimised individually to obtain the highest sensitivity. Multiplex PCR assays are complex to develop as primers and probes could have different optimal temperature requirements, binding between primers and probes could occur, affecting the performance of the PCR assays. At this stage, preference was given to Taqman assays, as these are a preferable option for multiplexing.

7.1.1 Aim

The aim of this study was to evaluate the feasibility of using DBS for the detection of life-threatening causes of fever in children. The methodology could be used by organisations and countries to implement a surveillance methodology.

7.1.2 Objectives

1. The possibility of a DBS based surveillance system for fever related illnesses in children was assessed by the following sub-objectives;
   a. To develop a method for detection of up to six pathogens on DBS
   b. To combine the detection methods into one streamlined process
   c. To perform an initial validation of the method with spiked and clinical samples
   d. To perform pilot studies in rural health care facilities

7.1.3 OUTLINE

To establish the possibility of a surveillance system as described above, the study took place at three locations: the development, experiments, and testing were performed in Leiden Cytology and Pathology Laboratory, in The Netherlands, and two field centres, one in Laos which provided already collected samples, and one field centre in Peru where the surveillance method was implemented.

Most attention was paid to the feasibility of the implementation of the method, collection of samples, extraction of nucleic acids, and detection of pathogens. Due to the technical nature of this laboratory based project, aspects as finances and public health impacts were not measured due to time and financial constraints.

7.2 Material and methods

7.2.1 *Pathogen selection*

The study focused on acute fever affecting children under ten years old. Pathogens were selected based on consultations of medical textbooks and journals, and on discussions held with various researchers and experienced clinicians who were active in one of our research locations. Feasibility with regard to the technical aspects of detecting the pathogens was also been taken into consideration. A final selection was made that fitted both the clinical criteria and technical feasibility.

As pathogens have different transmission routes and abilities to spread through the human body during infection, the ability to detect the pathogen by PCR heavily depends on pathogenic characteristics. Careful attention was therefore given to the selection of potentially suitable pathogens.

The criteria to select the pathogens were developed after discussions with researchers and experts at the London School of Hygiene and Tropical Medicine. A pathogen was considered eligible if the following criteria were met:

- Pathogen should cause fever in children
- Pathogen should be clinically important e.g. life threatening
- Pathogen should be detectable in blood
- Pathogen should be detectable by PCR
- Pathogens should be detectable during acute fever stage
- Pathogen should be potentially detectable in a drop of blood

Based on the list above, a large selection of pathogens could be included. To limit the selection, pathogens were reviewed based on prevalence at our research sites. The geographical distribution of pathogens was to some extent affecting the selection criteria as positive materials had to be obtainable within our collaborative network. The following pathogens were more closely reviewed:

Table 7.1: Details of selected pathogens

Pathogen	Type	Disease	Vector/transmission route
Plasmodium	protozoa	malaria	Mosquito
Dengue types 1-4	virus	dengue	Mosquito
Rickettsia typhi, Orienta tsustsugamushi	bacteria	murine and scrub typhus	Fleas – murine typhus Mites- scrub typhus
Leptospira pathogenic species	bacteria	leptospirosis/ Weil's disease	Water, food – urine/semen, animals
Chikungunya	virus	chikungunya	Mosquito
Bartonella bacilliformis	bacteria	bartonellosis/ oroya fever/ Carrion's disease	Sand flies

Although responsible for large epidemics, chikungunya virus is not usually life threatening to children. However, it would be potentially interesting to develop a suitable surveillance method, considering the recent outbreaks in Europe and South- East Asia (358).

7.2.2 Literature review

A literature review was performed to assess the range and performance of PCR based diagnostic methods on blood samples for pathogens related to acute fever with no localizing signs. The literature review was used to study pathogen load in blood, PCR performance and to select preferred PCR assays for our study. Pubmed was searched for suitable PCR assays. The search strategy is given in Annex 10.7.1.

7.2.3 Analysis of PCR assays

After the selection of potential methods from the literature, this study analysed the PCR assays to determine the theoretical capabilities of the assay before trying different assays in the laboratory.

Basic local alignment search tool (BLAST) was used as first analysis step (www.ncbi.nlm.nih.gov/BLAST/). By entering the nucleotides of the primers or probe, BLAST reveals which organism genomes match the nucleotides.

After BLAST, primer details were analysed by looking into homo / hetero-dimer capabilities of the primers. For this, we used an engine called; ITD primer (359).

The final analysis step was primer and probe alignment (Bio Edit sequence alignment editor software). The alignment results and the type of assay made up the final call. Once the assays were selected, laboratory tests were initiated. This process is described in Annex 10.7.2.

To determine the best PCR assay for a pathogen, a selection of pathogen isolates of high concentration were used for the initial comparison of assays. All preliminary experiments were performed on a Roche LightCycler 1.5 PCR machine (Roche Diagnostics, Basel, Switzerland). Melting curve analysis and 4% agarose gel electrophoresis (E-gel, Agarose gel, Invitrogen, UK) were used to determine specificity and primer-dimer susceptibility. Additionally, the performances of other primers targeting the same pathogen were compared by melt curve and DNA product analysis. After the probe was ordered, the laboratory steps as indicated above were repeated to visualise the binding of the probe and to determine whether the melt curve had the expected temperature.

7.2.4 *Analysis of PCR assays in the laboratory*

Strains were obtained to aid the PCR assay development and to compare the performance of assays against each other. Strains were provided by the Royal tropical institute (KIT) Amsterdam, The Netherlands, LSHTM, Institute of Animal Hygiene and Veterinary Public Health, Germany, School of Veterinary Science, Leahurst Campus, University of Liverpool, United Kingdom and Mahosot Hospital, Vientiane, Lao People Democratic Republic (Laos) (Table 7.2).

Table 7.2: List of species and strains used during this project

Strain	Origin
Plasmodium vivax	LSHTM
Plasmodium falciparum	LSHTM
Plasmodium ovale	LSHTM
Plasmodium ovale-mutant	LSHTM
Plasmodium malariae	LSHTM
Bartonella bacilliformis	University of Liverpool
B.bacilliformis NCTC12135	Health Protection Agency, UK
B.bacilliformis	University of Liverpool
B.bacilliformis	University of Liverpool
B.bacilliformis	University of Liverpool
Orientia tsutsugamushi	Mahosot Hospital, Laos
Rickettsia typhi	Mahosot Hospital, Laos
Leptospira interrogans	KIT Amsterdam
Leptospira santarosai	KIT Amsterdam
Leptospira weilli	KIT Amsterdam
Leptospira kirschneri	KIT Amsterdam
Leptospira meyeri	KIT Amsterdam
Chikungunya	Animal hygiene and Vet. Germany
Dengue 1 cDNA	KIT Amsterdam
Dengue 2 cDNA	KIT Amsterdam
Dengue 3 cDNA	KIT Amsterdam
Dengue 4 cDNA	KIT Amsterdam

The PCR assays were optimised by trying different primer and probe concentrations. Once the optimal concentrations were identified, pathogen concentration ranges were used to review the performance of the assay. If necessary, another substitute PCR was chosen at this stage.

Plasmid inserts were developed and ordered for the various pathogens in order to determine limits of detection, PCR performance, efficiency calculations, positive control and functions as positive DNA sequence for spiking blood samples. Plasmids were made and ordered by Genscript, USA. All sequences were inserted into pUC57 with EcoRV and protective bases were added to both ends. The following plasmids were based on the alignments obtained in the previous step. The plasmid sequences that were used can be found in Annex 10.7.3.

The following plasmids were developed:

Dengue types 1,2,3, and 4

Leptospira interrogans, L.weilli, L.santarosai, L. kirschneri, and *L. meyeri*

Plasmodium vivax, P. malariae, P. ovale, P. falciparum

Rickettsia typhi

Orientia tsutsugamushi

B.bacilliformis, mutant 1,2, and 1+2 combination

Chikungunya

The plasmids were eluted in Tris10 EDTA buffer and concentration series were made with Nucleic Acid buffer till 10^{-2} concentrations per µl. The concentrations of the plasmids were converted to number of copies by using Avogadro's number ($6.022^{*}\ 10^{23}$) employing this calculation:

Copies = X ng/µl * Avogadro's number / (length of basepairs * 1.10^{9} *mole bp).

PCR mastermix buffers were tested and compared to each other by running the same samples twice. We have tested the Quantitect SYBR green buffer (Qiagen, Hilden, Germany), Fast start universal SYBR green (Roche, Basel, Switzerland), Lightcycler 480 mix (Roche, Basel, Switzerland), multiplex PCR kit (Qiagen, Hilden, Germany) and the quantitect virus kit (Qiagen, Hilden, Germany).

All tests were performed at 50µl reaction volume instead of the more common 20 or 25µl reaction volume on the LightCycler 480 (Roche) (LC480). This was done to increase the detection limit and the potential for detecting the pathogens.

PCR efficiency is an overall indicator of the PCR run conditions by combining the quality of the mastermix, primer and probe conditions, and sample quality. A PCR efficiency is expressed on a scale from 0 to 2, of which a 2 indicates a perfect PCR amplification efficiency (by every cycle the DNA is copied 2 times) and a PCR efficiency of 0 indicates the PCR did not do anything (by every cycle the DNA is copied 0 times). To summarise, we applied

the rule of thumb to all PCR assays that when a PCR obtains an efficiency of 2, with a 10% lower value acceptance, we would not further optimise the assay.

7.2.5 DBS VALIDATION

Samples were spiked with blood, spotted onto filter paper with various concentrations of plasmids to determine the performance of the PCR in combination with DBS samples. Different spiking methods were applied to determine which would produce the least variability in the results. The three methods evaluated were (1) add plasmids and dilute blood, (2) add diluted plasmids to whole blood, and (3) add plasmids first, allow the cards to dry and then add whole blood to the filter paper. Different DBS extraction methods were compared within our laboratory. Different punching methods were applied among which scissors, hand punch and automated punch machine. Cleaning of the manual punches were done by dipping the punch into "RNAse away" solutions that contain nuclease enzymes that degrade DNA and RNA, followed by dipping into 96% alcohol and dried by punching through clean sheets of filter paper.

Every laboratory experiment was performed with a positive and negative control and performed in duplicate or quadruplicate.

7.2.6 SEQUENCING

To assess the specificity of the PCR assay, *B.bacilliformis* positives where sequenced. As our PCR amplifies a small segment (105bp), primers developed by Garcia-Esteban *et al.* were used for sequencing (360). PCR results were purified by enzymatic treatment of Exonuclease 1 (exol) and Alkaline Phosphatase (AP) both produced by ABI (ABI research, New York, USA). 5µl PCR product and 5µl mix (3.35µl H20, 0.5µl 10x AP dilution buffer, 0.15µl Exol and 1µl AP) were incubated for 60 minutes (min) at 37°C, 15 min at 72°C. Cycle sequencing reaction was performed according to manufacturer's instructions (Abi BigDye V3.1, 10µl reactions). The 96 well plates were cleaned by ethanol precipitation and a mixture (3µl 3M NaOAc, 62.5µl

absolute ethanol and 24.5µl H20) was added to 10µl sample. Plates were stored on ice for 20 mins, centrifuged at 3000g for 30 min and supernatant was discarded. 150µl of 70% ethanol was added and centrifuged at 3000g for 10 min. Supernatant was discarded and plates were left in the dark for 25 min. 10µl Hi-Di formamide was added to each sample and plates were loaded into the ABI sequencer (ABI 3730) (Applied Biosystems, Carlsbad, USA). Sequence data was analysed in Geneious basic v.5.6.4, (Geneious, Biomatters, Auckland, New Zealand).

7.2.7 *Extraction study*

The generous donation of FTAelute filter paper from GE healthcare for this project gave us the opportunity to work with these cards. The Lao study site was collecting DBS from patients with suspected typhus on FTAelute filter paper cards since 2010. As 100% cellulose filter papers (Whatman 903 or PerkinElmer 226) are commonly used in the detection of infectious diseases, an extraction study was performed to review which filter paper and extraction method would be preferable.

Three different types of cards and four extraction methods were compared. The M48 biorobot (MagAttract DNA kit Qiagen, Hilden, Germany), the blood DNA minikit spin columns (Qiagen, Hilden, Germany), Chelex (Sigma Aldrich, St Louis, USA), and the specific H2O extraction for FTAelute cards (Whatman GE healthcare, USA). Additionally to the FTAelute cards, the 903 filter paper cards (Whatman GE healthcare, USA) were used as well as the PerkinElmer cards 226 (PerkinElmer, Waltham, USA). An overview can be seen in Table 7.3.

Table 7.3: Provides an overview of extraction methods and filter paper cards used for the extraction study.

	Extraction methods			
Filter paper cards	**M48 biorobot**	**Blood DNA minikit**	**Chelex**	**H_2O**
Whatman 903	x	x	x	
PerkinElmer 226	x	x	x	
Whatman FTAelute				x

Filter cards 903 and 226 were spotted with 20µl buffer containing various concentrations of plasmids. Cards were dried and 50µl of fresh EDTA whole blood was added. Unfortunately, the same protocol led to incorrect extraction for the FTAelute filter paper samples. As the FTAelute filter paper itself contains impregnated lysis buffer, it became active when spotting the plasmids and was already dissolved when adding whole blood, leading to reduced lysis activity when whole blood was spotted. This affected the extraction, as the eluate contained hemoglobin, which is a PCR inhibitor. Filter paper where therefore spiked with 40µl whole blood containing the same amount of plasmids, per spot. The concentration of plasmids was kept the same for all types of filter papers. To make the study comparable, 6mm punch and final elution of 100µl was used for each extraction method. PCR input volume was the same for all types of filter paper and types of extraction platforms. The concentration that was added to the filter papers ranged from 4.5 c/µl of extracted sample to 450,000 c/µl. The measure of "c/ µl extracted sample" allows easy comparison between methods.

7.2.8 Clinical validation

The initial protocol was to establish a sample collection method in South-East Asia and allow paired testing on human sera or blood cultures to function as a reference standard for our surveillance detection methodology. Due to our South-East Asian collaborators' interests in this project and possibility of collaboration, we were able to obtain samples that were already collected during 2008-2010 and were collected from very well-defined clinically diagnosed patients. Obtaining ethical approval (in Ministry of Health, Laos and Oxford University, UK), setting up the study and enrolling patients had already been done by the collaborator. The reference PCR assays that were performed on the samples originating from Laos where based on assays published in peer-reviewed journals; malaria PCR performed on whole blood (184),, dengue PCR performed on serum (361), *R.typhi* (362) and *O.tsutsugamushi* where performed on buffy coat blood (363) and *leptospira* PCR was performed on whole blood samples (364).

Additionally, samples from the malaria reference center of the United Kingdom, located at the London School of Hygiene and Tropical Medicine, were used and analyzed.

Variability caused by using DBS and laboratory methods was addressed in detail throughout the study during the evaluation and selection of assays and comparison of extraction methods.

7.2.9 *Quality assurance*

For QA and control measures, human housekeeping genes for both DNA and RNA were used. β-actin for DNA control and GAPDH for RNA control were used. Additionally a quality check for DBS was developed based on the WHO guidelines (128). The DBS samples from Laos and Peru were visually checked and quality of cards was recorded per spot. Five criteria were used to categorise the quality of the spots (Annex 10.6). The humidity and overall quality of the card were recorded as well. Data was entered into a specially designed Epi-info file (CDC, Atlanta), to allow an easy linkage between PCR results and DBS quality. The record files developed for the studies performed in this thesis, have been posted online (354).

A DNA control was needed to support the evaluation of DBS samples. A human housekeeping gene was selected (β-actin) from the literature and the assay was performed on the LC480 (365). A sample needs to be positive for β-actin and have a lower cycle point (cp) value than 35. This cp value was chosen as a minimum due to the unlikelihood of finding pathogenic DNA in samples where there is almost no human DNA. A low level of human DNA indicates that the run or extraction has failed, or that not enough blood was spotted on the card.

To control the input volume of RNA, an assay targeting the human housekeeping gene encoding GAPDH was used (366). We applied the same rule of thumb as for the DNA human control that samples should have a lower cp value of 35. Well-prepared samples were used to determine the normal concentration of GAPDH to assure that a cp of 35 is acceptable.

7.2.10 *Implementation of the surveillance method in Peru*

The surveillance methodology was introduced in health clinics located in rural sites in Caraz, Ancash in Peru within the context of child health care services offered to patients with fever. All children with fever and under the age of 10 were eligible to participate in this study. Written consent of the parents or guardians was obtained before blood samples were collected. While patients at the study sites in Caraz were enrolled in 2010, an additional two healthcare clinics in Quillo and Yautan were included in 2011. Ethical approval was obtained from the ethical committees at the London School of Hygiene and Tropical Medicine and Universidad Peruana Cayetano Heredia.

7.2.11 *Bartonella*

This study was a unique opportunity to investigate detection of *B.bacilliformis* using DBS. *B.bacilliformis* is a bacterium that causes severe febrile episodes with often fatal outcomes. The bacterium enters the red blood cells and is capable of infecting up to 80-90% of all red blood cells, therefore causing acute anemia (43). If the infection becomes chronic it causes severe skin eruptions but the mortality rate decreases. It has been noted that the bacteria cause an epidemic every 5-7 years in young children in Caraz region, Peru (N.Solorzano, personal communication). To increase the possibility of detecting *B.bacilliformis*, we looked into all options to ensure our timing and methodology would be correct. In order to increase the likelihood of finding an epidemic, we initiated the following;

- Contacted the local and national surveillance authorities
- Determined best study sites to collect samples
- Located where earlier outbreaks have been found and analyzed
- Chose sites, based on past experience and outbreaks were first reported
- Chose the appropriate time of the year (rainy season)
- Took into account the likelihood of a 5 or 7 year interval between outbreaks.

7.2.12 Study procedures in Peru

At the beginning of the study, training was given to medical staff on how to obtain and store DBS samples correctly. The CDC guidelines regarding spotting, storing and mailing of DBS were used throughout the training (367). Stickers with specimen codes were placed on the DBS and on the patient's form before DBS were spotted with whole blood. DBS were spotted by holding finger or heel-prick blood against the filter paper. If venepuncture was necessary for other diagnostic tests, no finger pricks were performed but a small volume of venous blood was obtained to spot the DBS by pipette. Finger or heel prick were avoided whenever possible to reduce potential inconvenience for the child.

DBS racks were provided for overnight drying and in the morning the DBS samples were placed with two desiccants in a zip lock bag. The date, patient's number, and diagnostic test results for serology, blood culture or other diagnostic results were recorded on a standard form, using patient stickers to link the form with the DBS samples. This form was also used to record patient's admission date, symptoms, onset of symptoms and duration, to record variables that can influence the performance of the surveillance methodology. Additionally, the PCR results and clinical findings were combined to enable a better view of the patients' health and interpretation of diagnostic results. All the forms and training documents were translated into national languages. A visit to the health facility was made to thank participants, obtain feedback on any problems and feasibility of implementing DBS as a continuous surveillance method, were discussed. The samples were transported to Leiden Cytology and Pathology Laboratory, The Netherlands.

7.2.13 Statistical methods

The variability of cp values obtained by comparing the different extraction methods in combination with different filter paper types were summarised with box plots to display the median, 75th and 25th percentile and outliers of cp values.

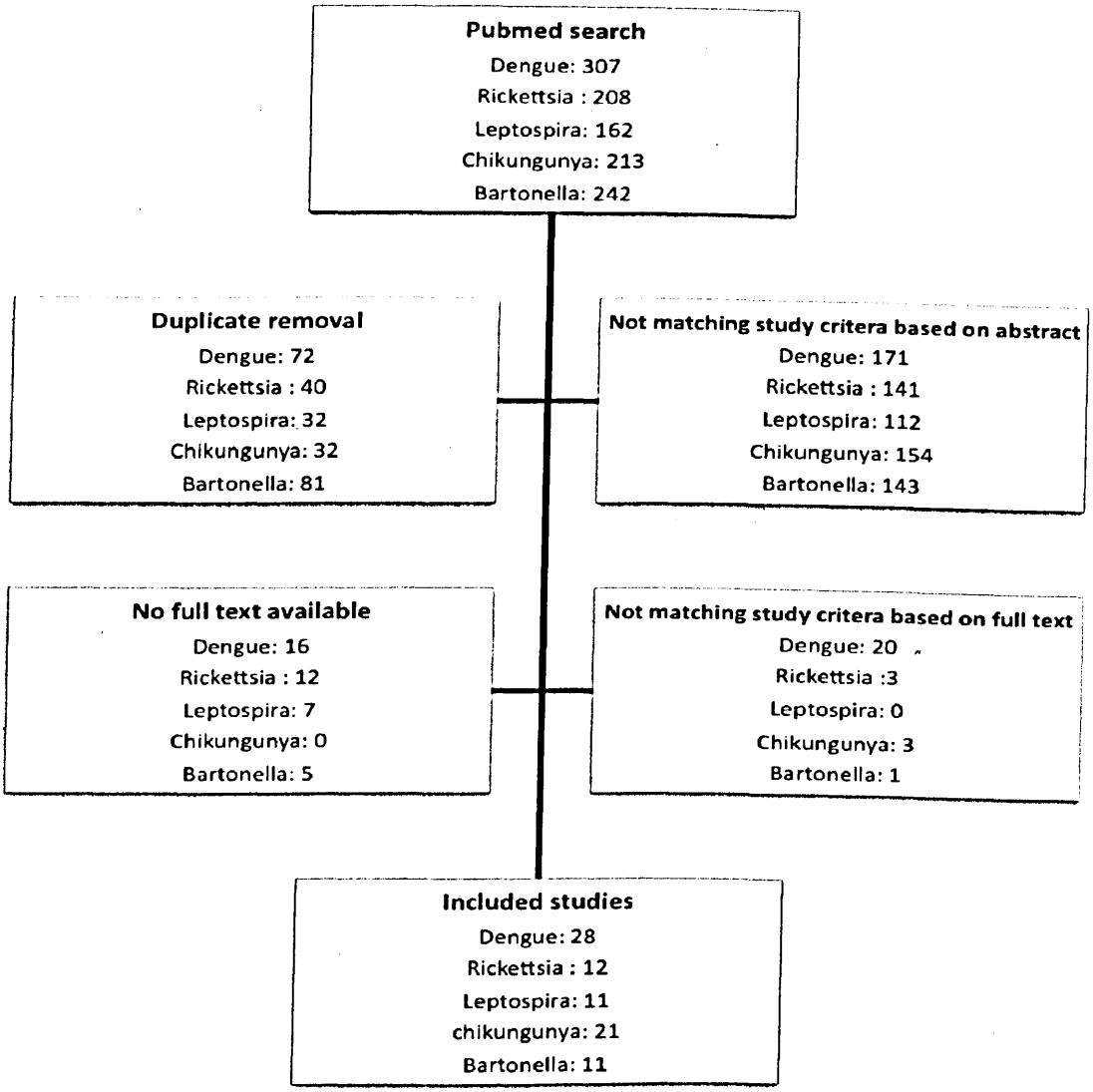
To review the likelihood of being *B.bacilliformis* positive based on self- reported symptoms, the symptoms were tested with Fischer Chi² test between the positive and negative *B.bacilliformis* group. Symptoms with p-value <0.20 were included for the binomial logistical regression model. High correlation between the symptoms was anticipated due to the close nature of various febrile illnesses and clinical presentation, which limited further evaluations. Akaike information criterions were used to select the variables in the multivariate analysis; general malaise, vomiting and diarrhoea were excluded from further analysis as they are not needed according to the Akaike information criterions). The binomial logistical regression model was used to identify symptoms that give patients a high probability (odds) of having a *B.bacilliformis* infection. Statistical software Stata 12 was used for the boxplots and statistical analysis.

7.3 RESULTS

7.3.1 *LITERATURE REVIEW*

Searches were performed to identify suitable PCR tests for every pathogen except Malaria. The search strategy is given in Annex 10.7.1

Figure 7.1: Flow chart of literature study, divided by pathogen. Numbers represent excluded or included number of studies



7.3.1.1 Malaria

No literature searches for malaria PCR assays were conducted due to the vast experience with malaria PCR at the school. The primers advised by malaria experts of LSHTM were used.

7.3.1.2 Dengue

The performance of a PCR assays greatly depends on the sample type and the timing of sample collection in relation to the patient's illness. Typically applied sample types for dengue detection include plasma (368), serum (369) and whole blood (370) or DBS samples (96). The sooner the sample was taken after the onset of symptoms appears to be of great importance for dengue detection (371). When a sample has been collected within 5 days after the onset of symptoms, plasma or serum samples gave good PCR results (371). Surprisingly, it appears that whole blood and DBS samples are more sensitive than plasma when collected after 5 days of fever (5, 95, 96, 370). A reason for this could be that dengue targets white blood cells, but no definite answer has been given.

The sensitivity of the PCR assays varied greatly between reference methods used. One assay obtained a sensitivity of 30.7% compared to an IgM assay (372), while most PCR assays performed on plasma samples detected 64% to 100% of dengue virus -positive samples (5, 95, 361, 368, 369, 371-386). Most assays made use of anti-dengue IgM as reference (377, 387), serology (388, 389), or the assay developed by Lanciotti (390-393). Although no official statements could be found, the nested PCR developed by Lanciotti *et al.* seems to be generally accepted as the gold standard. Specificity was in general high, ranging from 92% (394) to 100% (389). When the PCR assay consisted of multiple primers, the sensitivity varied by dengue strain from 64% for dengue 4 to 100% for dengue 1 (386).

In general, the detection limits of most PCR assays were excellent, ranging from 40 copies per ml to 100 copies per ml of whole blood (395). A few studies looked into quantitation of viral loads, which were reported to be up to 10^6 or 10^7 c/ µl (383, 396).

7.3.1.3 Rickettsia

Rickettsia are commonly divided amongst three groups; Spotted fever group (SFG), typhus (TG) and scrub typhus group (STG). SFG and TG are caused by various Rickettsia species, while STG is caused by *Rickettsia tsutsugamushi* which recently has been renamed to *Orientia tsutsugamushi*. One PCR primer pair that can detect both *O. tsutsugamushi* and *Rickettsia* spp have not been described. The PCRs that detect all *Rickettsia* species, instead of only specific species, also detect species that are not harmful to humans and could potentially bias the results found when analysing clinical samples. The aim is to detect TG and STG, while SFG is of less importance for the development of the surveillance method in Laos, as recommended by experts who participated in the pathogen selection process. In Laos, TG (as *R. typhi*) and STG are the most important rickettsial pathogens (397).

The various targets and PCRs developed for *Rickettsia* species indicate complexity in detecting the pathogen in blood related products. The sensitivity obtained by studies varied from 14% to 100% (398-403). The sample sizes of most studies are relatively low (average of 80 samples). The bacterial load detected in buffy coats or in serum where low (100-1000 c/ml) (404). A study detected the highest bacterial load in serum between 9-12 days (10^3 till 10^7 c/ml) (405). As for dengue, the sooner the samples are collected after the onset of fever, the higher the sensitivity. One paper suggested a completely different strategy of sample collection for *O. tsutsugamushi*. Within 5 days, the sensitivity was 0%, while between 9-12 days, the sensitivity went up to 100% (405). It will be extremely difficult to obtain high sensitivities with DBS since timing seems to be of great importance to obtain samples with high bacterial loads. It is unclear how exactly the pathogen bacterial load varies over time and how quickly it raises or declines over time. It seems that different types of sample materials (buffy coat, serum) influence the performance of the PCR assays. As *O. tsutsugamushi* is present in peripheral blood mononuclear cells, most assays used buffy coat samples while some used serum or EDTA blood (362, 363, 402, 406, 407).

7.3.1.4 Leptospira

The sample type varied between studies; serum (408-410), buffy coat (411, 412), whole blood (413), or unstated were used (364, 414). The amplified region as well as the assay method varied amongst the articles reviewed. Some assays only detected pathogenic species (406, 408) while some detected all types of *Leptospira* (413). *Leptospira* species are classified as pathogenic, indeterminate and saprophytic based on their potential effect on humans. Due to the diverse clinical manifestations, leptospirosis is highly underreported and is a major neglected tropical disease (415).

Although the specificity in general was 95-100% for all assays, the sensitivity varied from 29% up to 100% (15 samples) (408, 415). The contribution of sample selection and the reference methodology is believed to be essential for these varying results. It has been noted that within the first week of fever it is easier to detect *Leptospira* but sensitivity decreases when samples were collected after 4 days (411, 415).

The detection limits of the PCR assays according to the authors were good, ranging from 2 c/µl to 10 c/ µl which indicate the PCR works optimal (364, 408, 412, 416). One assay was capable to detect 10 leptospires/mL blood which was based on spiked samples and by using alive leptospires, not taking dead ones into account (408). Based on the limited data available, the amount of leptospires in blood will be low but there is a potential for being detectable by DBS.

7.3.1.5 Chikungunya

Serum and plasma samples were used to evaluate the performance of the PCR assays (368, 417-424). Some authors did not use a reference method for their developed PCR but used well-characterised panels (425, 426). The sensitivity and specificity of the assays were, if reported, 100% (419, 425, 427). The detection limits of the assays were very sensitive, around 5 copies/ PCR reaction (419, 422). The viral load in plasma appeared to be at its highest in the first 5 days of infection, as viral loads reached above 1 billion copies per mL of blood (425). Detection of chikungunya in acute febrile cases with smaller volume samples would be reasonably easy to be detected, according to the literature.

7.3.1.6 Bartonella

There are over 20 species of *Bartonella* of which 11 are known to cause disease in humans and animals. *B. henselae* is known to cause cat-scratch disease and various strains cause culture-negative endocarditis or trench fever. *B.bacilliformis* is believed to be the most deadly *Bartonella*, with a fatal outcome up to 88% during the acute phase for untreated patients (428). Our primary focus is *B.bacilliformis*.

The search for suitable real-time PCR assays was complicated and only two assays were real time (429, 430). Most assays target multiple B*artonella* species (430-433) or specifically *B.bacilliformis* (429, 434, 435). No data on bacterial load from clinical samples could be found in any of the selected articles.

7.3.2 *Theoretical detection capabilities*

To review the detection capabilities of the surveillance method, the theoretical detection capabilities were evaluated first. The detection of these pathogens depends on various factors which can be divided into three categories as depicted in Table 7.4.

Table 7.4: Variables influencing the performance of the surveillance method

Variables listed in three categories		
Pathogen characteristics	**DBS samples**	**Laboratory methods**
Quantity of pathogen in blood during infection	Correct collection of blood	DBS volume for assay (punch size)
Time pathogen is present in blood	Blood quantity collected	DNA/RNA isolation efficiency from DBS elute
Clinical presentation (fever) and active infection	Preservation of DNA/RNA	Quality of DNA/RNA isolated from sample
Infection differences between children or adults	Elution efficiency of DNA/ RNA out of blood	Removal of PCR inhibitors
Presence in plasma, serum, or blood cell components	In-correct storage effects on DNA/RNA	Target sequence (e.g. plasmid DNA)
Gram negative or positive bacteria		Isolated sample volume used for PCR
		PCR sensitivity

As noted in Table 7.4, various factors influence the sensitivity of the surveillance method developed in this proof-of- principle study. As this study aims to review the possibility of the method, potential limiting factors should be identified and addressed beforehand. As noted in the literature review, pathogen details and specific characteristics during infection cannot be found for many pathogens during infection or presence in blood.

Although most of these factors are discussed in Chapter 2 of this thesis, some factors are discussed in more detail below. The quantity as well as the period during infection of which pathogenic material is present in the blood, varies greatly per pathogen. In enteric fever, a few (1-10) bacteria per mL of whole blood are present, while for HIV there can be > million copies per mL (436). The limited data that is available for the selected pathogens is primarily based on clinical samples from adults. As infections in children often present differently than in adults, this limits the usefulness of this data. For example, malaria can be asymptomatic and potentially causes more severe infections in children. While malaria and Bartonella bacilliformis infect red blood cells, dengue infects white blood cells. This influences the composition and quantity of pathogen materials when extracting DNA or RNA from DBS samples instead of serum samples. As whole blood is applied centrally to the DBS spot, an uneven distribution of plasma and whole blood compounds can be expected on the filter paper (see Chapter 2 for more detail). Additionally, the laboratory steps and storage of samples influence the sensitivity of the assays applied. More details regarding these variables and information of using DBS samples can be found in Chapter 2.

To gauge the detection limits when using DBS, we looked at HIV as many studies have investigated quantifying HIV viral load. Based on these results, the limit of detection seems to range between 800-2,000 copies/ml (Chapter 3). As a detection limit for each of the pathogens has not been established for DBS samples, we used this limit as a guide.

To determine if the detection limit of DBS samples would be sufficient to detect pathogens within the first few days of infection, published pathogen loads in blood were reviewed (Table 7.5).

Table 7.5: Pathogen load in blood given by copies per mL (c/mL)

Pathogen	Pathogen load in acute cases	Reference
Malaria	$>10^5$ c/ml	(437)
Leptospira	10^3 c/ml	(438)
Dengue	$>10^5$ c/ml / 4.6log c/ml	(439) (440)
Rickettsia typhi	unknown	
Orientia tsutsugamushi	10^4 c/ml in buffy coat blood	(399) (404)
B.bacilliformis	unknown	
Chikungunya	10^4 c/ml to 10^9 c/ml	(425)

Variability between pathogens and patients are important. Based on the limited data available, it is likely that we could detect malaria spp, dengue, *B.bacilliformis* and chikungunya in patients during acute infection. For malaria, it is known that the infection can be detected successfully with DBS samples (441). This has also been demonstrated for dengue (5, 95, 370). *Leptospira* will be more difficult to detect as it will be likely that clinical cases could be below our detection limits and would be missed. No publication regarding the quantitation for *B.bacilliformis* in clinical samples could be found but based on results of blood smears and qualitative PCR assays, it is believed that acute cases could be detected with DBS (43, 44, 429).

The necessity for buffy coat blood samples for *Rickettsia* spp. and *O. tsutsugamushi* indicates that detection with normal whole blood would be difficult, if not highly unlikely. Buffy coat blood contains leukocytes and platelets and is obtained by centrifugation. After centrifugation, buffy coat blood accounts for less than 1% of the whole blood sample. Even though the pathogen load in buffy coat blood can be high (around 10,000 copies/ml), extrapolated to a non-centrifuged whole blood sample, the bacterial load will be very low. Even though it is difficult to obtain a reasonable sensitivity for *R. typhi* and *O. tsutsugamushi,* it would be a valuable effort to determine the feasibility, particularly due to the limited pathogen load data available.

The detection of dengue by DBS samples will potentially allow detection of acute disease, but will not be sensitive when sampled before or after acute infection. As noted, the dengue viral load can quickly drop after a few days of infection (372). This stresses the importance of accurate case definition, to correctly determine the sensitivity of an assay.

Unfortunately, it is not within reach of this proof-of-concept study to evaluate the pathogen levels in blood of febrile children. Additionally, assessment of the complex relationship of clinical presentation and bacterial/viral load in blood is not within reach of this study.

7.3.3 LABORATORY ASSAY COMPARISON

After the literature was read and summarised, the data were reviewed to find the most suitable PCR assays. The PCR assays were compared and analysed using BLAST searches, primers or probe characteristic analysis, and the alignment of primers as discussed in paragraph 7.2.4. Details are given in Annex 10.7.2. Based on these criteria, we discontinued underperforming assays for *Leptospira*, dengue and rickettsial pathogens (363, 364, 375, 381, 395, 405, 442). For malaria and chikungunya, no additional primers were evaluated as the primers of Mangold *et al.* and Laurent *et al.* performance fulfilled the criteria set in chapter 7.2.4. For *B.bacilliformis*, primers were developed in this study (Annex 10.7.2).

Below is given an overview of the assays that were finally selected after evaluating multiple PCR assays in the laboratory. The PCR settings that were used are also given (Table 7.6).

Table 7.6: Overview of assays used in this study, including PCR settings.

Target	Assay	Cycle conditions	For	Rev	Pr	Vol	method	mix
Malaria 18sRNA	Mangold *et al.*	95°C-10s, 50°C -5s, 72°C -20s +meltcurve	500	500	-	20	LC1.5	FastSTART DNA SYBR Green (Roche)
	This study	95°C -10s, 50°C -10s, 72°C -15s +meltcurve	500	500	-	50	LC480	FastSTART DNA SYBR Green (Roche)
Leptospira secY	Ahmed *et al.*	95°C-5s, 54°C -5s, 72°C -15s +meltcurve	400	400	-	25	Bio-Rad	iQTM SYBR Green Supermix (bio-Rad)
	This study	95°C – 10s, 54°C -10s, 72°C -15s +meltcurve	400	400	-	50	LC480	FastSTART DNA SYBR Green (Roche)
R.typhi ompB	Henry *et al.*	94°C -5s, 60°C -30s	200	200	300	25	Smart-Cycler	Separate consumables (Invitrogen)
	This study	95°C -15s, 60C°C -45s	400	400	200	50	LC480	LC480 mastermix (Roche)
O.tustsu-gamushi GroEl	Paris *et al.*	95°C-15s, 54°C -15s, 72°C-20s + meltcurve	200	200	-	20	Rotor-gene	Quantimix easy (Biotools)
	This study	95°C -10s, 54°C -15s, 72°C -20s +meltcurve	200	200	-	50	LC480	FastSTART DNA SYBR Green (Roche)
*B.bacilliformis ITS region**	Garcia Esteban *et al.*	94°C -30s, 64.3°C -60s, 72°C -90s	400	400	100	50	ecogen	Not reported
	This study	95°C -15s, 60°C -60s	500	500	250	50	LC480	Quantitect virus kit (qiagen)

Target	Assay	Cycle conditions	For	Rev	Pr	Vol	method	mix
Chikungunya 3'UTR	Laurent *et al.*	60°C -30min, 95°C -10s, 60C°C -30s	450	150	150	100	LC2.0	Taqman RNA amplification kit (applied biosystem)
	This study	50°C -20min, 94°C -15s, 60°C -45s	400	400	200	50	LC480	Quantitect virus kit (qiagen)
Dengue 3'NC	Leparc Goffard *et al.*	50°C -15min, 95°C -15s, 60°C -45s	200	200	100	25	rotor-gene	superscript 3 (Invitrogen)
	This study	50°C -20min, 94°C -15s, 60°C -45s	200	200	100	50	LC480	Quantitect virus kit (qiagen)

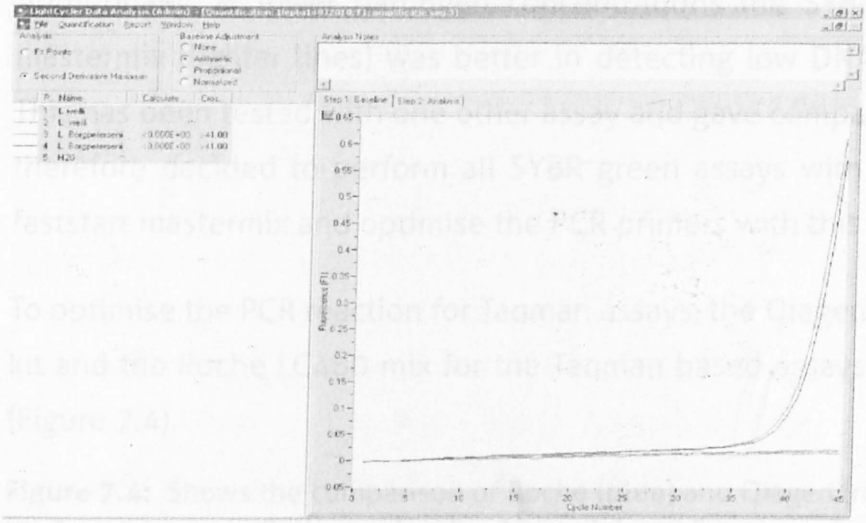
For= forward primer in nM, Rev = reverse primer in nM, prob = probe in nM, vol= volume in µl, Mg = Magnesium
*primers targeted the hypervariable intergenic transcribed spacer (ITS) located between 16S and 23S rRNA genes.

For dengue, 5 different assays have been tried and all were unsuccessful in detecting all 4 types of dengue. 2 assays could not detect all types, 2 assays could not detect low concentrations to an acceptable level and the last one required considerable amount of adjusting before it could be used (361, 375, 377, 395). In order to do this, plasmids have been ordered and different mixes, primer and probe concentrations have been used. Surprisingly this did not alter the behaviour of the assay at low concentrations of dengue, in which the curves transformed into straight 35 degree lines instead of the traditional curves. In the end, samples were sent for dengue PCR tests to KIT Amsterdam. The dengue PCR that was used by KIT, was published by Menting *et al.* in 2011 (443). Except for dengue, all other assays were performed at LCPL laboratory in Leiden, The Netherlands.

7.3.4 *Determine best PCR mixes*

We have tested different PCR buffers as Quantitect SYBR green buffer (Qiagen, Hilden, Germany), and Fast start universal SYBR green (Roche, Basel, Switzerland), Lightcycler 480 mix (Roche, Basel, Switserland), Qiagen multiplex PCR kit and the Qiagen quantitect virus kit. The most striking difference in mix was found with the Quantitect SYBR green mastermix and the Roche Fast start universal SYBR green mix. The result obtained with the Qiagen SYBR green mastermix mix and Roche Fast start universal SYBR green mix is given below, indicating a better performance of the Roche Fast start universal SYBR green mix. The evaluation was performed with *Leptospira* PCR and various *Leptospira* species.

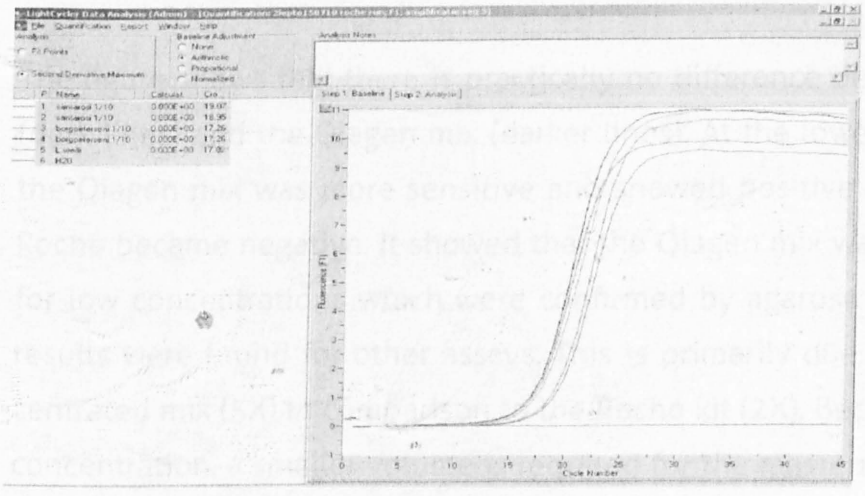
Figure 7.2: The performance of two PCR master mixes, above is Qiagen SYBR green master mix, below is Roche SYBR green mix. X-as is cycle number and horizontal fluorescent level per Leptospira pathogen.



Both runs were performed on the same day, same PCR machine and same sample material. The poor performance of the Qiagen Quantitect SYBR green mastermix has been discussed with technical representatives of Qiagen and runs were repeated several times. The Qiagen kits were refunded due to the lack of any suitable explanation by Qiagen technicians.

The same comparison was done for the SYBR green mix. We compared the Roche SYBR green faststart mix, initially developed for the Lightcycler 1 and 2 series, and the Lightcycler 480 SYBR green mastermix.

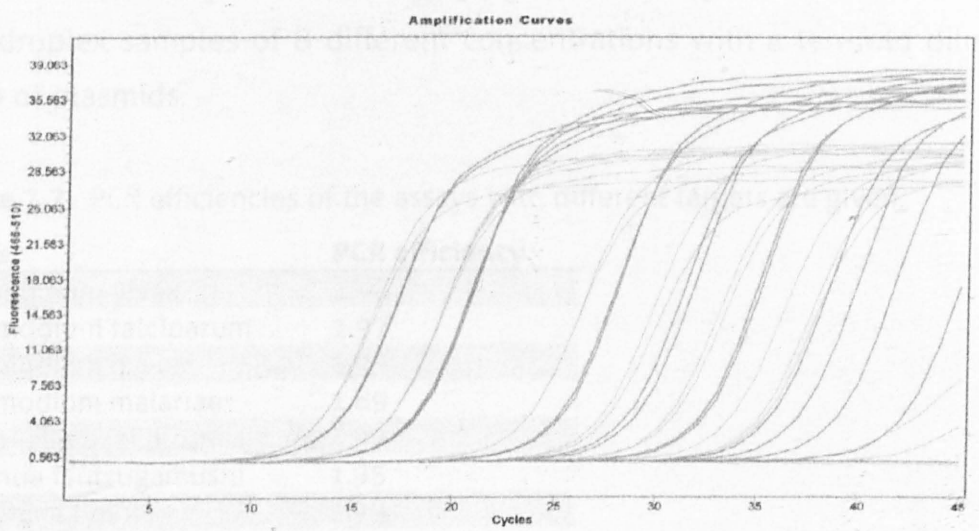
Figure 7.3: Comparison of two Roche PCR SYBR green master mixes with the same sample input and same primer concentrations. PCR cycle numbers horizontally and fluorescence are given vertically with various malaria DNA concentrations.



The SYBR green faststart mastermix performed better than the Lightcycler 480 SYBR green mastermix as lower cp values were obtained (Figure 7.3). Additionally, at lower pathogen concentrations the SYBR green faststart mastermix (lighter lines) was better in detecting low DNA concentrations. This has been tested with one other assay and gave comparable results. We therefore decided to perform all SYBR green assays with the SYBR green faststart mastermix and optimise the PCR primers with this mastermix.

To optimise the PCR reaction for Taqman assays, the Qiagen quantitect virus kit and the Roche LC480 mix for the Taqman based assays were evaluated (Figure 7.4).

Figure 7.4: Shows the comparison of Roche (blue) and Qiagen (red) master mixes for Taqman assays. Cycle number (horizontally) and fluorescence (vertically) are given with various B.bacilliformis DNA concentrations as sample.



The figure shows that there is practically no difference between the Roche (dark lines) and the Qiagen mix (darker lines). At the lower concentrations, the Qiagen mix was more sensitive and showed positive results while the Roche became negative. It showed that the Qiagen mix was more sensitive for low concentrations which were confirmed by agarose gel. Comparable results were found for other assays. This is primarily due to the high concentrated mix (5X) in comparison to the Roche kit (2X). Because of this high concentration, a smaller volume is required for the mastermix which makes it possible to increase the patient sample volume used for the PCR reaction.

This potentially produces a greater sensitivity. Even though this mastermix was initially developed as a Reverse transcriptase, we decided to use this assay also for DNA targets to standardise the procedures as much as possible.

7.3.5 PCR EFFICIENCY

As the final primer and probe concentrations as well as the PCR mastermixes were determined, experiments to determine the PCR efficiencies where performed. PCR efficiency is an overall indicator of the PCR run conditions by combining the quality of the PCR machine, the mastermix, primer and probe conditions, and sample quality. A PCR efficiency is expressed on a scale from 0 to 2, of which a 2 indicates a perfect PCR amplification efficiency (by every cycle the DNA is copied 2 times) and a PCR efficiency of 0 indicates the PCR did not do anything (by every cycle the DNA is copied 0 times). The PCR efficiency calculation is based on two separate runs, both with quadruplex samples of 8 different concentrations with a ten-fold dilution step of plasmids.

Table 7.7: PCR efficiencies of the assays with different targets are given.

PCRs	PCR efficiency
Plasmodium vivax	1.98
Plasmodium falciparum	1.97
Plasmodium ovale	1.95
Plasmodium malariae	1.89
Bartonella bacilliformis	1.97
Orientia tsutsugamushi	1.95
Rickettsia typhi	1.99
Leptospira interrogans	1.99
Leptospira santarosai	1.95
Leptospira weilli	1.95
Leptospira kirschneri	1.96
Leptospira meyeri	1.98

PCR efficiency is a valuable measure of performance of a PCR assay. A value of 2 is optimal performance.

Different species and strains (see Table 7.1 for details) were tested per PCR assay as variability in efficiency can occur.

7.3.6 PCR ASSAY AND DBS VALIDATION

Since the efficiencies of the PCRs were within the acceptable range established at the beginning of the study, the sensitivities of the assays were determined by using plasmids with known concentrations. Every plasmid concentration was run 8 times and at least in two runs, giving the performance in Table 7.8.

Table 7.8: Total of positive outcomes of 8 samples tested with different concentrations

PCR	# of samples detected with different plasmid concentrations per µl					
	10^3	10^2	10^1	1	10^{-1}	10^{-2}
Plasmodium vivax	8/8	8/8	8/8	8/8	5/8	2/8
Plasmodium falciparum	8/8	8/8	8/8	8/8	4/8	1/8
Plasmodium ovale	8/8	8/8	8/8	8/8	6/8	0/8
Plasmodium malariae	8/8	8/8	8/8	7/8	5/8	2/8
Bartonella bacilliformis	8/8	8/8	8/8	8/8	6/8	4/8
Orientia tsutsugamushi	8/8	8/8	8/8	8/8	2/8	0/8
Rickettsia typhi	8/8	8/8	8/8	8/8	8/8	0/8
Leptospira interrogans	8/8	8/8	8/8	8/8	8/8	3/8
Leptospira santarosai	8/8	8/8	8/8	8/8	6/8	1/8
Leptospira weilli	8/8	8/8	8/8	7/8	6/8	3/8
Leptospira kirschneri	8/8	8/8	8/8	8/8	6/8	1/8
Leptospira meyeri	8/8	8/8	8/8	8/8	8/8	2/8

For the RNA assays, plasmids were ordered to validate and optimise the assays but were not used to determine the lower limit of detection of these assays, because this would exclude the efficiency of transforming RNA into DNA (reverse transcription) before the PCR reaction occurs.

7.3.7 SPIKING BLOOD SPOTS

The plasmids were eluted in a 1:100 elution with EDTA whole blood. 40µl was used to spot the cards and were dried overnight. The protocol of the filter paper manufacturer was slightly adjusted to improve the performance of extraction of FTAelute filter paper. A 6mm punch was used instead of 3mm, 1ml was used to wash the punch, and an extra wash step was added and the total elution volume was 100 µl. Table 7.9 highlights the results of two PCR reactions performed in quadruplicate for each of the plasmids.

Table 7.9: Total of positive outcomes of 8 samples tested with different plasmid concentrations of dried whole blood

PCR	# of samples detected with different plasmid concentrations per µl of whole blood spotted on DBS (FTAelute)				
	10^6	10^5	10^4	10^3	10^2
Plasmodium vivax	8/8	8/8	8/8	8/8	8/8
Plasmodium falciparum	8/8	8/8	8/8	8/8	8/8
Plasmodium ovale	8/8	8/8	8/8	8/8	8/8
Plasmodium malariae	8/8	8/8	8/8	8/8	8/8
Bartonella bacilliformis	8/8	8/8	8/8	8/8	8/8
Orientia tsutsugamushi	8/8	8/8	8/8	8/8	8/8
Rickettsia typhi	8/8	8/8	8/8	8/8	8/8
Leptospira interrogans	8/8	8/8	8/8	8/8	8/8
Leptospira santarosai	8/8	8/8	8/8	8/8	8/8
Leptospira weilli	8/8	8/8	8/8	8/8	8/8
Leptospira kirschneri	8/8	8/8	8/8	8/8	8/8
Leptospira meyeri	8/8	8/8	8/8	8/8	8/8

No plasmids series were spiked in DBS for the RNA targets (dengue and chikungunya). Plasmids are DNA structures and the results could therefore not be translated to RNA, as the amplification efficiency can be very different.

7.3.8 *Extraction study*

Although we could not change filter paper because all our positive samples from Laos were already spotted on FTAelute, a comparison study was set-up with different kind of cards and extraction methods to obtain insight in how FTAelute compares to other filter papers.

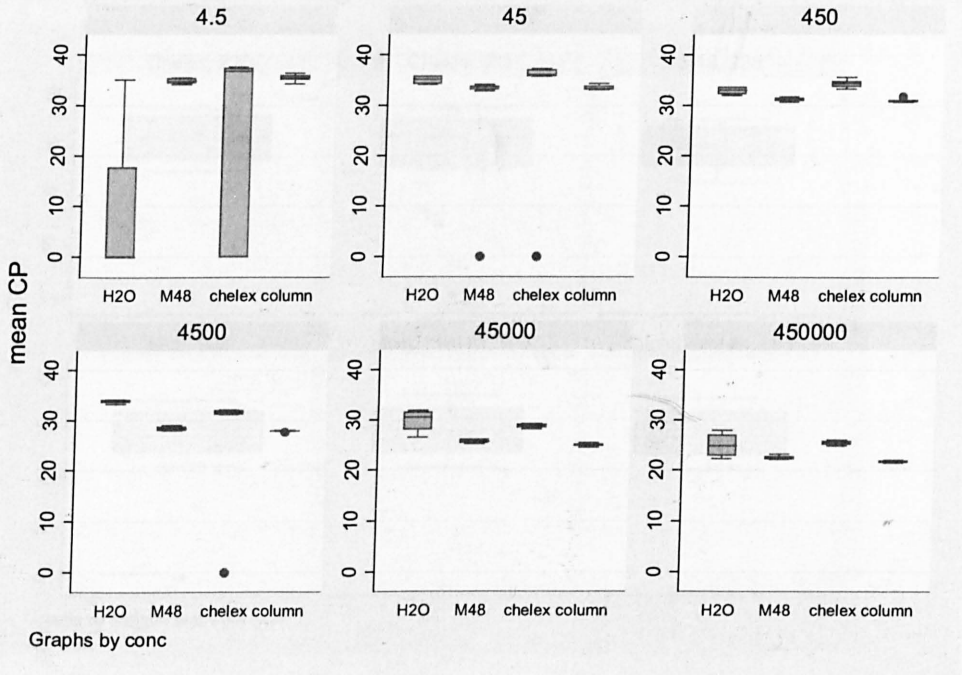
Because the detection limits of the different PCR assays did not show much variability (Table 7.8), we selected one assay (*B.bacilliformis*) and determined the extraction efficiency with only this assay instead of repeating this for the numerous pathogens.

The mean cycle point (cp) given for every extraction method was used as a measure of extraction efficiency. A lower cp value means a higher concentration detected in the PCR. In theory, all extraction methods should provide the same cp value since all methods used the same samples. The M48 refers to the automated M48 BioRobot of Qiagen, the DNA blood minikit (Qiagen) is a spin column based extraction (referred to as column) and the Chelex extraction (Bio-Rad, Veenendaal, Netherlands) is a manual and more affordable option than the blood mini kit. H_2O extraction can only be performed with the FTAelute cards.

The concentrations of spiked samples are given as concentration per µl extract (4.5 copies/µl to 450,000 copies/µl). The concentration values above the tables would be obtained when the extraction obtains 100% extraction efficiency.

Figure 7.5: The box plots show the performance of the different extraction methods given per concentration of DNA per spot, with mean cp on Y-axis.

The median, 75 percentile and 25 percentile of cp values are given in a box with the upper and lower whiskers representing the spread of the highest or lowest 25% of cp values.



Outliers are given as dots. The smaller the boxplot, the less variance in results, suggestive of very minor variability in results. Cp of 0 means no amplification was detected, suggesting an incorrect extraction. At 4.5 copies/µl, H20 and chelex extraction often failed, which causes the boxplots to be very wide. The three negative samples (2 at 45 copies/µl and 1 at 4500 copies/µl), were potentially caused by extraction defaults.

For Figure 7.5, the concentrations of spiked samples are given as concentration per µl extract (4.5 copies/µl to 450.000 copies/µl) that would be obtained with 100% extraction efficiency. The M48, chelex, and column results were based on 903 and 226 filter paper.

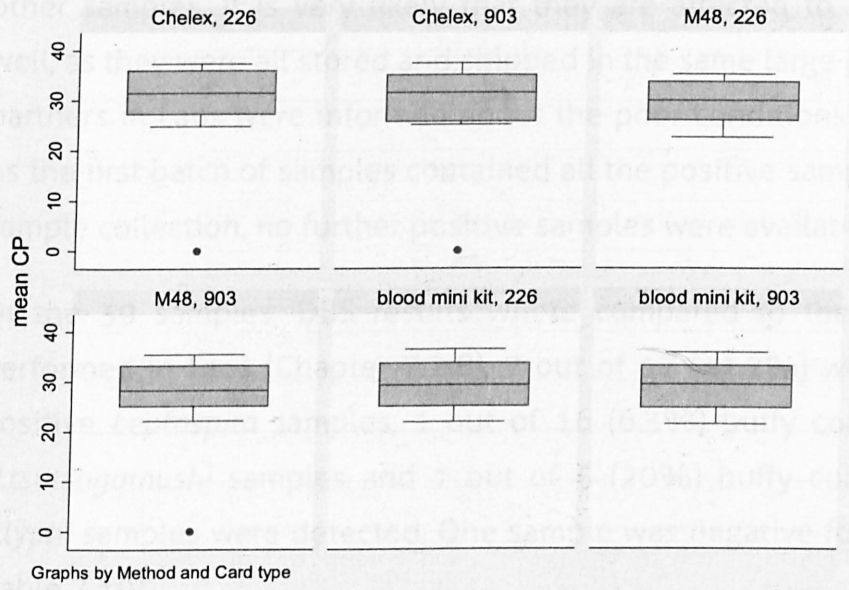
The Chelex and H2O methods were not always successful in extracting the lowest concentration of 4.5 c/µl which caused the boxplot to be very wide. The small variation in results found by the M48 and blood mini kit indicates a robust extraction performance throughout different plasmid concentrations. The FTAelute cards showed increased variability when plasmid con-

centrations were high.

To evaluate if there was a difference between the two 100% cellulose filter paper cards, the 903 Whatman and 226 of PerkinElmer were evaluated (Figure 7.6).

Figure 7.6: The extraction performance given per cycle point (Cp) per method and card type.

226 = PerkinElmer 226 filter paper, 903= Whatman 903 filter paper. The median, 75 percentile and 25 percentile of cp values are given in a box with the upper and lower whiskers representing the spread of the highest or lowest 25% of cp values. Outliers are given as dots.



There seems to be no major difference between the 903 and 226 filter papers which could be expected since technically, there is no difference in the material of the cards.

7.3.9 CLINICAL SAMPLES

A total of 122 FTAelute filter paper card samples were mailed from Laos to the laboratory in the Netherlands and were tested for all pathogens. Upon arrival of the samples, the quality of the DBS samples were checked (procedure as given in chapter 5). In total, 64 samples (52%) were unfortunately invalid due to humidity (n=39, 61%) or presence of serum rings (N=25, 39%). All samples were dried and extracted for a human DNA quality control PCR test (β-actin). A total of 39 (32%) samples passed the criteria of 35 cp. Even though these samples were less affected by poor sample storage than the other samples, it is very likely that they are affected to some degree as well, as they were all stored and shipped in the same large plastic bags. Our partners in Laos were informed about the poor conditions of the samples. As the first batch of samples contained all the positive samples from 3 year sample collection, no further positive samples were available.

Of the 39 samples, DBS results where compared to the reference test performed in Laos (Chapter 7.2.8). 7 out of 17 (41.2%) whole blood PCR positive *Leptospira* samples, 1 out of 16 (6.3%) buffy coat PCR positive *O.tsutsugamushi* samples and 1 out of 5 (20%) buffy coat PCR positive *R.typhi* samples were detected. One sample was negative for all pathogens (Table 7.10).

Although DBS samples are commonly used to diagnose malaria, we initiated a small evaluation to see if our methodology would work with clinical samples. A selection of malaria samples were obtained from the malaria reference laboratory based at the London School of Hygiene and Tropical Medicine. We used samples from Africa that were: *Plasmodium ovale, P. falciparum, P. vivax, P. ovale* for evaluation of the performance of the methodology. There was 100% concordance between DBS PCR techniques and PCR on whole blood. As the principal investigator was not blinded to these results, we could not include these samples to estimate the sensitivity. We obtained malaria positive DBS samples from Vietnam to provide an estimate of the sensitivity of the methodology. Unfortunately, no chikungunya positive clinical samples could be obtained that were spotted onto filter paper from any of our contacts.

Table 7.10: Overview of the detection performance given per pathogen by our methodology

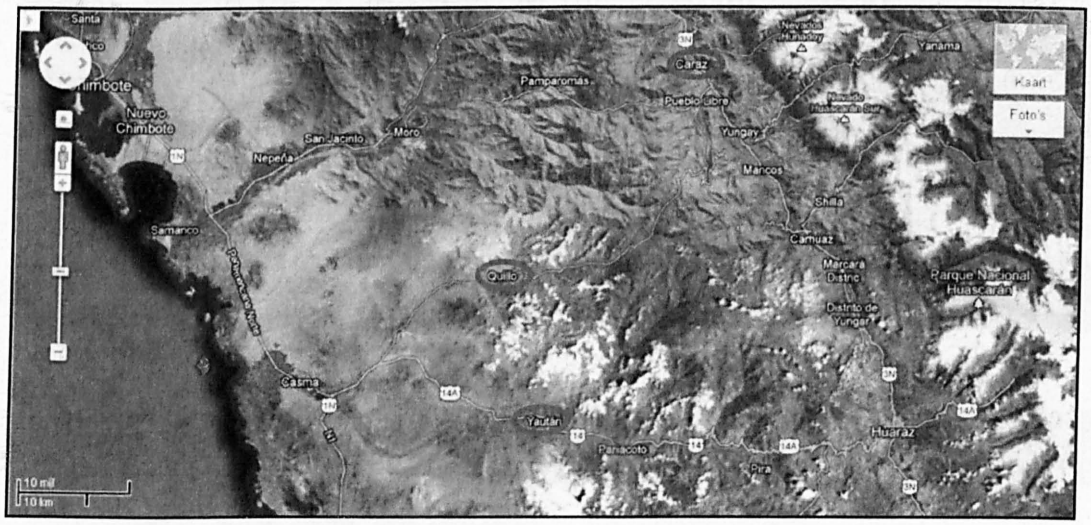
Pathogen	# detected	Total	Detection rate %
Leptospira	7	17	41.2
O.tsutsugamushi	1	16	6.3
R.typhi	1	5	20.0
Dengue	9	68	13.2
Malaria	5	5	100

7.3.10 Implementation of the surveillance method in Peru

The surveillance study was established in Ancash province, Peru to evaluate the performance of detecting *B.bacilliformis* by DBS samples and secondly, to analyse the implementation of the surveillance method.

A total of 196 cards were collected in Caraz, Ancash Peru between November 2010 and February 2011. In September 2011, an expedition was organised in a different district called Yuatan, Ancash province to search for signs of latent *B.bacilliformis*. Based on the results of the expedition, we included 2 clinics in Yuatan district, the main hospital in Yuatan and Quillo (Figure 7.7). Clinics in Caraz area were given additional materials to collect samples should a *B.bacilliformis* outbreak occur during the 2012 rainy season. However, there was no outbreak and no additional samples were collected in Caraz. In Yuatan district, 65 samples were collected in Yautan and Quillo health centres from December 2011 till July 2012.

Figure 7.7: Map of Ancash province. Caraz, Quillo and Yautan are highlighted (source; Google maps)



7.3.11 *Quality of the cards*

Upon arrival in The Netherlands, samples were checked for humidity and placed in new ziplock bags and stored at 4°C until further processed. Out of 262 samples, 119 (49.3%) were invalid according to our quality assessment protocol, of which most incorrect samples were collected from Caraz and Yuracoto clinics. The poor quality of the cards was discussed and appeared to be caused by use of finger pricks with a small width and depth. These finger pricks were already used in the Peruvian clinics and were not supplied by this study. New lancets were purchased and transported to the clinics. When the new lancets were used, sample quality improved greatly as only 31 (26%) incorrect DBS samples were collected instead of 88 (74%) before the new lancets were used. The humidity of the cards was checked upon arrival at the laboratory which showed that 124 (63.3%) DBS samples were dry and 72 required change of desiccants when they arrived in The Netherlands. Table 7.11 provides an overview of the samples collected in Peru.

Table 7.11: Overview of samples obtained from Peruvian clinics

	Caraz hospital	Mato clinic & outreach	Yuracoto clinic	Yautan hospital	Quillo clinic	Total
Start collection	2010	2010	2010	2011	2011	
# samples	87	53	57	32	33	262
Male (%)	50 (57%)	35 (66%)	30 (53%)	14 (44%)	17 (52%)	164 (63%)
Age (range)	3.37 (0-10)	6.23 (0-11)	3.33 (0-10)	2.35 (1-10)	4.38 (1-8)	4.03 (0-11)
Average Temp (range)	37.52 (36-39.5)	38.46 (37-40)	37.98 (36-39.8)	38.27 (37-39.2)	38.40 (37.5-39.5)	38.01 (36-40)
Average days of fever (range)	2.48 (1-8)	2.02 (1-7)	1.49 (1-4)	2.41 (1-5)	2.39 (1-5)	2.07 (1-8)
Invalid samples (%)	41 (47.1%)	31 (58.5%)	41 (71.9%)	6 (18.8%)	0 (0%)	119 (49.3%)
Blood smear positive	0 (0%)	0 (0%)	0 (0%)	1 (3%)	1 (3%)	0 (0%)
DBS-PCR positive	1 (1%)	0 (0%)	0 (0%)	1 (3%)	15 (46%)	17 (6%)

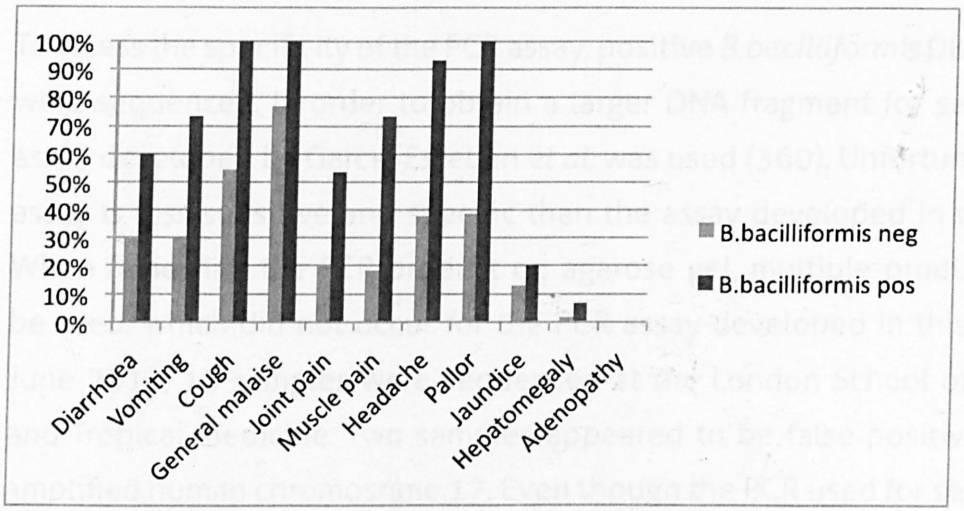
7.3.12 CLINICAL FINDINGS

The febrile children participating in this study were young, with a mean age of 4 years (Table 7.11). The length of fever before samples are collected is an important variable that can affect the sensitivity of diagnostic assays as bacterial loads are likely to be lower during recovery. Additionally, days of fever are a suitable measure to review the social behaviour of visiting health centres and provide a measure of reviewing the accessibility of healthcare. The data indicates that most febrile children were brought to the health centres within 1-3 days of onset of fever (n=234, 89%), indicating a positive trend towards early health seeking behaviour and accessibility of health centres. The temperature of children upon arrival at the clinics showed that 93 children (35%) had temperature below, and 169 (64%) children had temperatures above 37.0°C.

From each of the infants or children enrolled in the study, a DBS sample and blood smear was obtained. 2 blood smears were called positive while 17 samples were positive with PCR on DBS samples. The two positive blood smear results were of children from Quillo region who suffered from anaemia (Hb <3 gm/dl) and had high densities of *B.bacilliformis* in their bloodstream, according to the PCR results (>100,000 copies/DBS spot). This was also visible, as DBS spots had serum rings and blood spot was light coloured.

Almost 70% of the children sampled in 2010 had general malaise and coughing as main symptoms and were diagnosed with upper respiratory tract infections. In Figure 7.8, the symptoms of children who were *B.bacilliformis* PCR positive (n=17) and children without *B.bacilliformis* infection detected (n=245) are plotted in percentages.

Figure 7.8: Symptoms of children in given as percentages.



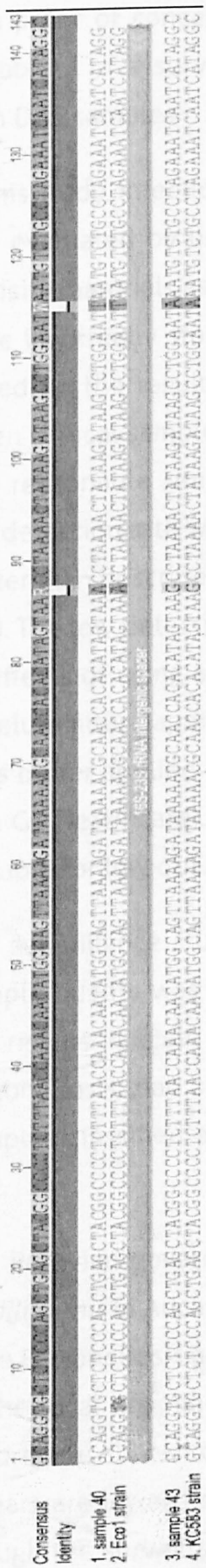
Clinicians could record multiple symptoms per child. Neg=negative, pos = positive.

To assess if diagnosis based on clinical symptoms would be feasible, we analysed separately the symptoms between *B.bacilliformis* positive and negative patients, to select symptoms with a P <0.20 (Fischer chi² test). A binomial logistic regression model was used to assess the association between symptoms and *B.bacilliformis* infection. The symptoms that had a Fischer chi² test of p <0.20 were included in the multivariate model. This selection process and AIC (Akaike information criteria) aids in minimalizing the variables in the model, excluded general malaise, vomiting and diarrhea. Even though the number of *B.bacilliformis* positives are small, patients during the rainy season of 2011/2012 in Peru with pallor and muscle pain had a high probability (odds) of being *B.bacilliformis* positive (binomial logistic regression, odds ratio (OR) 18.1,p= 0.007 (pallor) and OR 4.5 p= 0.01(muscle pain)). Even though this suggests that patients with these symptoms are more likely to be positive for *B.bacilliformis*, it cannot be used for diagnosis of patients due to limited representativeness (i.e. one region) and small number of samples included in the study.

7.3.13 SEQUENCE RESULT

To assess the specificity of the PCR assay, positive *B.bacilliformis* DBS samples were sequenced. In order to obtain a larger DNA fragment for sequencing, assay developed by Garcia Esteban *et al.* was used (360). Unfortunately, this assay is less sensitive and specific than the assay developed in this study. When reviewing the PCR product on agarose gel, multiple products could be seen, which did not occur for the PCR assay developed in this study. In June 2012, 10 samples were sequenced at the London School of Hygiene and Tropical Medicine. Two samples appeared to be false positive as they amplified human chromosome 17. Even though the PCR used for sequencing was not developed to differentiate *B.bacilliformis* strains, the remaining 8 samples were positive for *B.bacilliformis,* seven samples matched strain KC583 and 1 matched eco1 *B.bacilliformis* strain (Figure 7.9) (NCBI accession numbers: DQ179107 *Bartonella bacilliformis* strain EC01 and CP000524 *Bartonella bacilliformis* KC583).

Figure 7.9: Sequence result of two samples matching two strains (KC583, Eco1)



7.4 Discussion

This proof of concept study was initiated to evaluate the possibility and suitability of a surveillance method by combining nucleic acid detection with DBS samples

In this study, infectious diseases were detected by PCR and were developed and evaluated by using plasmids, whole genome of viruses, bacteria and parasite materials as well as clinical samples. Unfortunately, clinical samples were incorrectly stored which affected the detection of pathogens greatly. Based on the results obtained from spiked samples, results suggest that, when clinical samples are collected appropriately, it may be possible to obtain reasonable sensitivities. As expected for *R.typhi* and *O.tsutsugamushi*, the detection rate was low but the detection of dengue was expected to be better than described in the literature (13% compared to 81.6% sensitivity) (95). The low detection rate of clinical dengue samples was primarily linked to effects of using a type of filter paper inappropriate for RNA viruses. The FTAelute filter paper that was used is not intended for RNA detection as the lysis buffer applied to these cards disintegrates RNA (personal communication GE Healthcare). Additionally, the extraction method seemed to be less suitable for low quantities of viral or bacterial DNA/RNA in clinical samples.

The surveillance study was easily implemented at the health centres and sample quality was good when the correct finger prick lancets were used. The results indicate that DBS PCR method was more sensitive for detecting Bartonellosis than blood smears, as confirmed by sequencing. The only two samples classified as positive by blood smear reading were confirmed by PCR.

The Peruvian government has set-up a surveillance system for *Bartonella bacilliformis* in Ancash province. Blood smears of febrile patients thought to have Bartonellosis are collected in clinics and sent to the laboratory located at the local hospital where screening takes place. In case of an outbreak, reports are sent directly to the Ministry of Health. The sensitivity of blood smears are lower than those of the method developed in this study. Even though the surveillance method is used, clinical signs are more commonly

used as indicators for Bartonellosis cases than blood smears. Because of this, the blood smears have limited functionality (43).

7.4.1 LIMITATIONS

This proof of concept study was developed to highlight the possibilities and limitations of surveillance by using DBS. It was not intended to develop and validate all aspects of a surveillance method.

The number of clinical samples was low and unfortunately, the samples from Laos were too wet to reliably be tested. This particularly raised the importance of developing adequate protocols to obtain good quality specimens. Correct storage information was shared and implemented in Laos. Additionally, no positive samples for Chikungunya could be obtained which limited the evaluation.

To sequence the *B.bacilliformis* positive Peruvian samples, a different assay, which was less sensitive and specific than the assay developed in this study, was used to obtain larger sequences. This complicates the interpretation of the two negative sequence results. The two false negative samples could be true positive samples but this was potentially not visible given the larger quantity of amplified human DNA. These two samples had, when visualized on agarose gel, many DNA products. Suggestively, by cutting out the band for sequencing, the human chromosome was extracted and not the thinner *B.bacilliformis* band. In the future, it would be worthwhile to perform whole genome sequencing or develop an assay to genotype *B.bacilliformis* in greater detail.

Discussion

Chapter 8

8 Discussion

8.1 Main findings

The overall aim of the thesis was to assess whether DBS could improve patient management in remote settings. Here, the main findings are listed, followed by limitations of the study and recommendations for the future.

8.1.1 DBS for detection of infectious diseases

This thesis highlighted the feasibility of DBS to be used for the detection of various infectious diseases, based on the available literature. From humans, almost all types of body fluids, from blood to saliva and faeces to breast milk, have been stored on filter paper. When using DBS it is inevitable that the processed sample volume is small compared to gold standard samples as long as there are sufficient levels of the target molecules are contained in a DBS, the use of DBS improves patient access to state of art technologies that they would otherwise be denied.

DBS can be used to improve access to HIV VL measurements in remote settings, even though the sensitivity can in some cases be 0.5 log lower than that for plasma samples. Depending on the threshold for switching to a second line regimen, this loss of sensitivity may be tolerated if the use of DBS allows timelier switching to an appropriate and effective therapeutic regimen for HIV. The systematic reviews shows that key information was often not reported and there were frequent methodological variations such as in sample collection, storage, processing of filter paper and data analysis (chapter 3).

Standardization of evaluation methods is essential to improve the use of DBS in public health. For reporting DBS evaluation studies, this thesis provided an updated STARD checklist with the inclusion of specific DBS related information that should be reported when publishing evaluation studies (Chapter 2).

8.1.2 *DBS AS QA METHOD FOR POCT*

Syphilis serology using DBS as samples proved to be highly sensitive and specific compared to plasma samples. Although the method is robust and accurate, it relies on visual interpretation of agglutination, which is subjective. An increase in sensitivity was obtained when technicians were more experienced in interpreting DBS TPPA agglutinations, stressing the importance of training, particularly for subjective tests such as TPPA.

The QA method was implemented to assess the quality of DBS samples and the quality of POCT testing over a period of ten months in Tanzania. The collection of DBS by HCW in Tanzania was easily rolled out and the collection, storage and transportation proved to be good, giving high quality samples. The study showed that it is feasible to monitor test quality in field sites with DBS. Compared to the dried test method developed by the US CDC, DBS gave a more accurate picture of the quality of testing and was useful in identifying the need for training or refresher training. Like DTS, it is possible to carry out QA for both HIV and syphilis using a single DBS sample (Chapter 6).

8.1.3 *DBS FOR SURVEILLANCE FOR CAUSES OF FEVER*

Laboratory protocols were developed to enable the detection of seven pathogens from DBS. PCR assays were developed and evaluated by using plasmids, whole genome of viruses, bacteria and parasite materials, and clinical samples. Based on the results obtained from spiked samples, it was possible to obtain reasonable sensitivities for malaria, dengue, *leptospira*, and *B.bacilliformis*. *R.typhi* and *O.tsutsugamushi* are difficult to detect with DBS due to the very low number of pathogens in the blood stream. Unfortunately, many of the archived clinical samples were incorrectly stored which affected the detection of pathogens greatly. High quality clinical samples could only be obtained for *B.bacilliformis*. Using DBS with an in-house *B.bacilliformis* PCR method resulted in a more sensitive method for the detection of *B.bacilliformis* than the standard diagnostic test used in Peru. Positive results were confirmed by sequencing results. To our knowledge, this is the first validation of a *B.bacilliformis* PCR assay with DBS, and the assay showed excellent sensitivity and specificity.

8.2 The role of DBS in patient management in the future

The applicability and potential benefit of using DBS in areas such as the detection of infectious diseases, surveillance and quality assurance has been highlighted in this thesis. However, with the development of novel POC diagnostic methods that can be used at remote areas in developing countries, the use of DBS may be no longer needed in the future. Our studies suggest that the use of DBS may remain useful to assure the quality and improve accessibility of diagnostic tests in remote settings. As long as diagnostic assays rely on high quality laboratory infrastructure and are technically demanding, the usefulness of DBS to improve accessibility of those tests for remote settings remains. It is highly unlikely that POCTs will replace syndromic management for all infectious diseases in the near future. DBS will remain useful for determining the changing aetiology of syndromes and for disease surveillance as long as collection of blood samples on filter paper remains easy and affordable.

There is potential for filter paper to become more than just a sample collection matrix. Filter paper is becoming a diagnostic test itself. Microfluidic paper-based analytical devices have already been applied to HIV and syphilis detection and showed comparable results to traditional laboratory based assays (444, 445). In a few years' time, it is expected that these filter paper based tests are ready for clinical use and when this happens, diagnostics will change dramatically. Microfluidic papers are an exciting development with great potential to improve patient management in both developing and developed countries.

8.3 Recommendations

8.3.1 *Recommendations for standardisation*

This thesis provided a general guideline for collecting, storing and processing DBS samples in the laboratory, for NAAT or serological testing. These guidelines can be adjusted according to specific requirements depending on the disease syndrome or the diagnostic assays. Ideally, protocols should be developed in close collaboration with manufacturers, to assure high quality protocols are accessible for every laboratory using that platform. This will aid harmonisation of protocols and in obtaining consistency in test results between laboratories. Key concerns and the need for standardisation in terminology and methodology for DBS were addressed in this thesis, and will be published so that they are widely disseminated amongst those who work in global health.

The quantification of RNA in DBS is technically challenging. HIV VL protocols will be useful for other viruses as well, particularly when quantification is necessary. The work of HIV VL on DBS samples in this thesis should ultimately aid in the development of standardised protocols for the use of DBS for other infectious diseases, particularly when low quantities of RNA or DNA need to be detected.

8.3.2 *Recommendations to start using DBS samples*

This work shows that DBS used with PCR is a feasible and highly sensitive alternative to the use of blood smears for the surveillance of *B.bacilliformis*. Epidemiological and cost-effectiveness studies are required before they can replace Giemsa stained blood smears.

The significant variability of syphilis POCT results identified at the ten clinics highlight the necessity for a QA method to be implemented alongside POCTs. More attention should be given for the development and implementation of QA methods specifically designed for POCTs. While proficiency panels are regularly used as QA method for POCT, the work in this thesis showed that they are of limited use, compared to DBS QA, in detecting underperforming clinics that require remedial training. In this work, the QA method was devel-

oped for POCT used in PMTCT services in Tanzania but could be extrapolated to other infectious diseases and settings. There is a considerable potential in collecting DBS to retest samples from remote clinics or for epidemiological studies.

When DBS samples are used instead of plasma, clinicians and laboratory technicians should be aware of potential differences in results, particularly for HIV VL measurements when using the WHO recommended threshold of 5,000 c/ml for changing to second line treatment. As DBS adds minor variability in HIV VL results, false positives or false negative outcomes are more likely to occur than with plasma samples. Although certain aspects of DBS HIV VL measurement require further investigation, the use of DBS remains the only viable means of improving access to HIV VL for monitoring HIV patients on treatment.

8.4 LIMITATIONS

The limitations of each of the studies are discussed in the chapters individually. In retrospect, there are certain aspects that I have learned that would affect the design and execution of this type of study in the future.

First, no data was obtained to monitor which HCWs collected the samples in the QA study. Monitoring which HCWs collected the samples can be useful to review the potential impact of training on the quality of the samples and the test results. Additionally, this data would have been useful to assess the attrition and rotation of HCWs. Within our research setting, where the study relied on the willingness of the HCWs to participate in this study, it was not feasible to track which HCWs provided the QA data.

Second, the average time to get samples from the QA study sites in Tanzania to the laboratory in the Netherlands was 95 days, which exceeds the recommendations made in chapter 2. By increasing the number of shipments to The Netherlands, this could have been improved. Due to financial and staffing constraints to visit all ten clinics for sample collection (takes at least four days) and shipping the samples to the Netherlands, this was not feasible in our study. The ideal scenario would be for the QA DBS samples to be processed in the National Instititute for Medical Research in Mwanza on a monthly basis and the results reported to Geita district for any corrective action, if necessary.

Third, the process of collecting and distributing data between the laboratory and clinics is an essential part of QA programs. Although an epi-info file and excel sheet were developed to potentially transform the QA data into useful figures for supervisors to monitor the performance and see trends in the quality of POCT, it was not within reach of the study to evaluate the process of collecting, distributing, and interpreting the QA data, due to time and staffing constraints.

Fourth, for the surveillance study, certain choices were made that, in retrospect, potentially affected the outcome of the study (Chapter 7). Laboratory tests could have been performed at another laboratory with validated PCRs for most of the infectious diseases included in this study. Given that

this study was part of a PhD and logistically and financially dependent on the laboratory where testing took place, it was not feasible to make use of another laboratory. Unfortunately, due to financial and time constraints we could not take this study further and actively collect samples in more research settings than in Peru.

Fifth, there were two occasions related to performing research abroad that influenced the study results. Even though these occasions where minor, the effects were substantial. The samples from Laos were ruined by a small mistake in not bringing samples from the freezer to room temperature before repackaging, causing condensate to be trapped inside. Study protocols and guidelines should have been distributed, even when collaborators have already collected the samples. The other occasion was in Peru, when samples were collected by HCW in Caraz. As they already obtained blood by finger prick, we did not distribute our finger prick lancets. Unfortunately, the finger prick lancets used in Caraz had a small needle that gave not enough blood for DBS, which caused the samples to be of poor quality. Before the study initiated, efforts could have been made to assess the quality of the lancets that were already used in Peru for spotting DBS samples.

Sixth, since the positive *B.bacilliformis* cases were sampled in January 2012 and confirmed to be positive in June by sequencing, we were not able to investigate this outbreak in greater detail. It is unfortunate that the positive cases were detected only in the extended study period as this limited the possibilities to obtain more funding to pursue our investigation.

8.5 FUTURE RESEARCH

Before the QA method can be implemented on a larger scale, costing studies should be performed. The costs of QA, as well as the costs of training, lot testing, and the costs of the tests itself, should be included to calculate the program costs. A QA method for POCT is necessary and as shown in this study, proficiency panels alone are not enough to indicate clinics where remedial training is necessary. Because QA is not merely the collection and testing of DBS samples, but also the translation of QA data into useful monitoring data for supervisors, this thesis initiated the development of suitable tools for processing and managing data. It is recommended that this process is evaluated before DBS QA is implemented.

For syphilis serology on DBS, the protocol developed in this study did not provide a solution for indeterminate TPPA test results. The thesis suggested, based on hands-on experience, that retesting in duplicate seemed suitable to establish a final reading. In the future, appropriate procedures for indeterminate results should be developed.

A rigorous approach would be required to extend the development of a surveillance method for febrile children. The main areas that would require attention are: clinical case definition, selection of pathogens, details of pathogens in the blood stream during febrile periods, and harmonisation of the methodologies into a high throughput method. Selecting the pathogens for the surveillance method could be done by using, for example, the Plex-ID developed by Abbott diagnostics, USA. This PCR and mass- spectrometry combination can differentiate undefined bacterial and viral infections by using broad spectrum PCR. This aids in prioritizing pathogens that are frequent, preventable and treatable. When a list of infections is made, it is worthwhile to determine the pathogen characteristics and quantity in blood during febrile illness to support future development of diagnostics and surveillance methods. This information will aid in validating the method in greater detail by using spiked samples that represent actual clinical situations. Clinical case definitions are indispensable as the duration of infection and the moment of collecting a sample profoundly influences the bacterial/viral load in blood. As seen in Peru, many febrile infants did not have a high fever which

influences the chance of finding positive cases. The average number of days before seeking medical aid after the onset of fever was relatively low, but this varies per region and country. Efforts to develop protocols for multiplex reactions or techniques such as Luminex (Life Technologies, UK) would be worthwhile to increase the number of pathogens being detected from DBS. A surveillance method based on serological markers could be worthwhile to assess.

This thesis assessed the use of DBS for three aspects of patient management. It might be worthwhile to assess the potential of DBS for other aspects, as outbreak investigations, or drugs and vaccine research development.

8.6 Conclusion

The goal of this research was to determine whether DBS can improve patient management in developing countries. The potential of a simple and reliable sample collection method such as filter paper was shown in this thesis. While DBS is not the best sampling method, it is the simplicity and robustness that makes it useful in remote settings. This thesis has shown the benefits and weaknesses of using DBS for the detection of infectious diseases in remote settings. More specifically, the utility of DBS in respect to three aspects of patient management have been discussed; first, to make laboratory based diagnostics more accessible for patients in remote settings, secondly, to assure the quality of diagnostics used at the point of care, and thirdly, for the surveillance of febrile illnesses in children.

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10 Annexes

10.1 Annexes to Chapter 1

No annexes for Chapter 1

10.2 Annexes to Chapter 2

10.2.1 Review protocol

Review of the clinical use of filter paper in infectious diseases
Draft Protocol

Review Team

Pieter Smit, Ivo Elliott, David Mabey, Rosanna Peeling, Paul Newton

Review Objectives

Primary Objective

To assess the use of dried blood spots (DBS) for the diagnosis or surveillance of infectious diseases.

Secondary Objective

To assess the general implications of using DBS compared with traditional sampling methods

Tertiary Objective

To assess the use of filter paper for diagnosis or surveillance for samples other than whole blood

PICOS

INCLUSION CRITERIA (PICOS)

Population

Studies evaluating the use of DBS as a replacement of a gold standard sample (plasma, serum, whole blood obtained by venous puncture, buffy coat blood, etc.) for any infectious disease diagnostic or surveillance assay.

Interventions (index tests)

Any commercially available technology for surveillance or for diagnosis of infectious diseases with DBS samples.

Reference standard/comparators

The same commercially available technology for the diagnosis or surveillance of infectious diseases with reference samples (plasma, serum, whole blood etc), when applicable.

Outcomes

Include studies evaluating DBS for quantitative as well as qualitative analysis of nucleic acid testing against plasma samples (DNA / RNA).

Include studies evaluating DBS for Antibody Antigen detection as diagnostics, cut-off determination, and protocol development (in combination with commercially available assays).

Study design
Evaluation studies published in peer-reviewed literature. Conference abstracts and letters to the editor were excluded.
Other
English language only, 1980-present.

Identifying research evidence

Search strategy

Databases: MEDLINE and EMBASE

MEDLINE: All aspects of clinical medicine, biomedicine, nursing, dentistry, allied health, health policy, genetics etc. Emphasis on English-language source, quite biased towards journals published in N. America. Includes journal articles from 1950s onwards and is updated daily.

EMBASE: All aspects of clinical medicine, biomedicine, nursing, dentistry, allied health, health policy, genetics etc. Quite biased towards information in journals published in Europe. Particularly strong on pharmaceutical information. Includes journals from 1947 onwards and is updated weekly.

Search Terms

Search terms for MEDLINE and EMBASE:

Ti,ab = keyword (title and abstract)

Mp= keyword (free text)

1. "dried blood".ti,ab
2. "blood spot*".ti,ab
3. DBS.mp
4. "filter paper".ti,ab,
5. "Guthrie filter".ti,ab
6. "filter cards".ti,ab
7. "dried blood spot*".ti,ab
8. "FTA*".ti,ab
9. "Flinders Associates Technology"
10. "Guthrie paper". Ti,ab
11. "dried serum*".ti,ab
12. "serum spot*".ti,ab

13. "filter card*".ti,ab
14. "filter disc*" .ti,ab
15. "filter disk*" .ti,ab
16. "blotting paper".ti,ab
17. "Guthrie card".ti,ab
18."Isocode stix".ti,ab

The following disorders and tests are commonly performed using DBS samples and were excluded: congenital hypothyroidism, phenylketonuria, cystic fibrosis, sickle cell disease, Tay-Sachs disease, haemoglobinopathies, galactosidase, spinal muscular atrophy, isovaleric acidaemia, maple syrup urine, cholesterol, triglycerides, HbA1c, human growth hormone, insulin, mercury. Other key words often associated with the search terms but not relevant to this review were also excluded: MRI, MR, CT, PET, N-glycan, Pharmacokinetic, Pharmacodynamic.

Date

1980 to present

Language

English

Publication type/status

Published works in peer reviewed journals

Study selection

Stage 1: Screening of titles/ abstracts against inclusion criteria.

Titles and abstracts, where available, will be screened and either accepted, rejected as not relevant, or rejected due to failure to meet inclusion criteria (if so, the reason will be specified).

Stage 2: Full papers obtained and assessed against inclusion criteria. Papers will be either accepted or rejected due to failure to meet inclusion criteria and the reason will be specified.

Full papers will be independently assessed by TWO members of the review team and results will be cross-checked and combined.

Inclusion Criteria

- Evaluation or comparison of performances of commercially available DNA/RNA assays with DBS and reference sampling methods
- Evaluation or comparison of performances of commercially available Antibody/Antigen assays with DBS and reference sampling methods
- Any human pathogen
- Evaluations based on human clinical or reference materials

Exclusion criteria

- Not an evaluation study or not having a correct reference sample or reference method
- In-house developed assays
- Full article not accessible
- Studies with other primary aims other than evaluation of DBS with reference samples (but keep these articles for objective 2)
- Studies related to drug resistance screening, genotyping, sequencing, other "non-diagnostics" evaluation studies
- Studies using filter paper for non-whole blood samples (urine, stool, cervical swabs, serum, plasma, saliva, cerebrospinal fluid, etc) (but include in objective 3).
- Any non-infectious diseases included in neonatal screening programmes (e.g. Galactokinase deficiency, cystic fibrosis

Data Extraction

General Information
Date of data extraction
Identification features of the study:
Record number
Author
Article title
Citation
Study Characteristics
Aim/ objectives of the study
Disease
Filter paper type
Participant/ Sample Characteristics

Characteristics of population from which samples were drawn:
Age
Sex
Number of samples
Sample country/ region of origin
Sample type (finger prick/ EDTA/..)
DBS storage:
At research site
At laboratory
Type of reference sample
DBS quality check performed?
Technology
Name and manufacturer of assays under evaluation
Extraction method
Extraction volume/adjustments
Extraction kit/ method used
Detection method
If quantitative; adjusted for DBS sample input?
Outcome data/ results
Unit of assessment/ analysis
Outcomes:
NA:
Mean pathogen load ± standard deviation
Range of pathogen load
Correlation (r)
Bias (mean difference)
Sensitivity, cut-off
Specificity, cut-off
% CV index test, reference test
Serology:
Sensitivity, cut-off
Specificity, cut-off
Cut-off adjusted for DBS?
Titre lower limit of detection
For each pre-specified outcome:
Reported (Y/N)
Definition used in study
Additional outcomes reported
Details of any additional relevant outcomes reported

Objective 2: To fulfil the second objective of this systematic review, the following information will be collected from publications in combination with our experience:

All articles excluded for objective 1 are reviewed that fulfil the criteria:

- Provide a better understanding of DBS samples regarding; card types, filter paper characteristics, collection, storage, extraction, environmental effects, stability, punch methods, cost compared to reference methods, transportation, policy & regulations, recommendations made by leading organisations (WHO, CDC), and other related topics that could improve understanding of filter paper]

Data extracted:

General Information
Date of data extraction
Identification features of the study:
Record number
Author
Article title
Citation
Assay details
Disease
DBS Topic
Results
Key findings

Objective 3: To fulfil the third objective of this systematic review, the following information will be collected from publications:

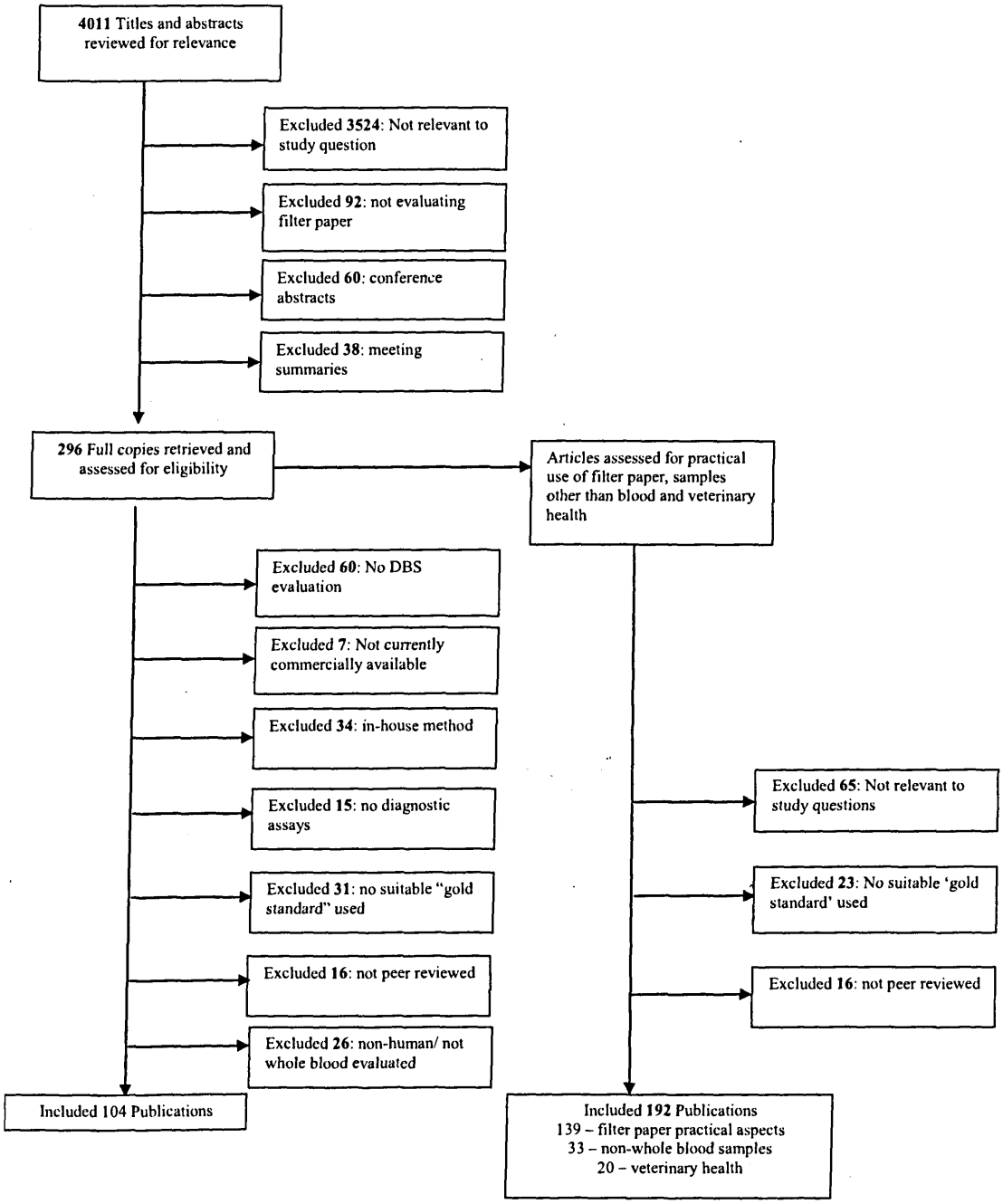
All articles that are excluded for objective 1 and 2 can be included.

- Make use of alternative samples stored on filter paper such as; serum, plasma, stool, urine, CSF, etc

Data extracted:

General Information
Date of data extraction
Identification features of the study:
Record number
Author
Article title
Citation
Study details
Disease
Sample type
DBS Topic
Assay details
Extraction method
Detection method
Results
Sensitivity
Specificity
Key findings

10.2.2 FLOW CHART OF INCLUDED AND EXCLUDED STUDIES



10.2.3 Summary of studies evaluating DBS for HIV and HTLV1

Disease, Assay type & country	Author	Number of samples & filter paper type	Test	Sensitivity %	Specificity %	Notes %
HIV 1& 2 serology Sierra Leone*(131)*	Boillot *et al. (1997)(131)*	359 samples Whatman No. 3	Innotest (diagnostics pasteur)	100 (HIV1) 87.5 (HIV2)	100	DBS 2.3 times cheaper than venous blood collection (Boillot *et al.*)
India	Lakshmi *et al. (2007)(134)*	225 samples Whatman No. 3	Murex Ag/Ab ELISA (Murex Biotech)	100 (HIV1)	100	HIV 1&2. Also tested on 12,617 patients for seroprevalence with overall sensitivity 99.6% and specificity 99.9%, based on subset of samples evaluated with PCR. (Lakshmi *et al.*)
HIV 1 serology Brazil*(132)*	Castro *et al. (2008)(132)*	457 samples Whatman 903	Q-Preven HIV1+2 (Symbiosis Diagnostika)	100	99.6	
Rwanda	Chaillet *et al. (2009)(446)*	491 samples Whatman 903	Uni-Form 2 (Abbott) & HIV-1.2.0 (BioMerieux)	100	99.1	
Zimbabwe	Mashange *et al. (2003)(135)*	379 samples Whatman No.3[5]	Gel Particle Agglutination HIV1&2 (Serodia) & Recombigen HIV1&2 ELISA (Trinity Biotech)	100 !00	98.7 99.6	

Disease, Assay type & country	Author	Number of samples & filter paper type	Test	Sensitivity %	Specificity %	Notes %
The Gambia	Sarje-Njie *et al. (2006)(136)*	200 samples Whatman BFC 180[6]	ICE HIV1.0.2 ELISA,(Wellcozyme) HIV recombinant (Murex), ICE HIV2 ELISA GPA	NR	NR	Pooling of 5 DBS samples would not lead to any missed positives. (Sarje-Njie *et al.)*
India	Solomon *et al. (2002)(137)*	89 samples Whatman 903	HIV1&2 (Serodia), Pepti-LAV ½ (Sanofi) , Micro-ELISA (Vironostika)	100	100	OD declined after 6 days storage at 36.8C & 70% humidity. (Solomon *et al.*)
HIV 1 serology (p24) Dominican Rep, USA, Vietnam, Malawi	Cachiafeiro *et al.(2009)(138)*	617 samples Whatman 903	Modified Up24 Ag (Perkin Elmer)	94.4	100	Storage of >30 months, decreased sensitivity to 72.2% (39/54 samples) (Cachiafeiro *et al.*)
Tanzania Switzerland	Knuchel *et al. (2007)(139)*	282 samples Whatman No.3	HIV p24 Ag (Perkin Elmer)	84	100	Non subtype D sensitivity: 93% Non subtype C sensitivity: 94% (Knuchel *et al.*)
Malawi	Mwapasa *et al. (2010)(140)*	222 samples	ELISA Alliance HIV1 p24 without ELAST (Perkin Elmer)	84	98	Study only included clear positive and negatives. (Mwapasa *et al.)*

Disease, Assay type & country	Author	Number of samples & filter paper type	Test	Sensitivity %	Specificity %	Notes %
South Africa	Patton *et al. (2006)(142)*	141 samples Whatman No.1	Ultrasensitive p24 Ag ELISA (Perkin Elmer)	98.8	100	Only children included: 34 days – 12yrs old. After 6 weeks storage, sensitivity dropped to 88.9%. (Patton *et al.* 2006)
South Africa	Patton *et al. (2008)(141)*	246 patients Whatman No.3 & no.1	Ultrasensitive p24 Ag ELISA (Perkin Elmer)	96.6 (W. No.1) 98.3 (W.903)	100 100	Desiccant improved sensitivity for storage >6 weeks. (Patton *et al.* 2008)
HIV 1 NAAT (DNA) Thailand*(143)*	Leelawiwat *et al. (2009)(143)*	162 samples Whatman 903	Amplicor HIV-1 DNA v. 1.5 PCR (Roche)	100	100	Same results for manual and automated methods (Nsojo *et al.*)
Tanzania	Lofgren *et a.l (2009)(144)*	176 samples Whatman 903	Cobas AmpliPrep/ TaqMan DNA (Roche)	97	100	On restesting false negatives, sensitivity increased to 100% (Patton *et al.*)
Tanzania	Nsojo *et al. (2010)(145)*	325 samples Whatman 903	Amplicor HIV-1 DNA v1.5 PCR (Roche)	98.3	99.6	
South Africa	Stevens *et al. (2008)(147)*	206 samples Whatman 903	Amplicor HIV-1DNA v1.5 PCR (Roche)	98.3	100	
South Africa	Patton *et al. (2007)(146)*	300 samples Whatman No.1	Amplicor HIV DNA v1.5 PCR (Roche)	100	99.6	
South Africa	Sherman *et al. (2005)(130)*	800 samples Whatman 903	Amplicor HIV-1 DNA v1.5 PCR (Roche)	99	99.8	

Disease, Assay type & country	Author	Number of samples & filter paper type	Test	Sensitivity %	Specificity %	Notes %
HIV1 NAAT (RNA) Thailand*(143)*	Leelawiwat *et al. (2009)(143)*	162 samples Whatman 903	manual NucliSENS (BioMerieux)	100	100	HIV 1. VL also measured and was 0.4 log lower in DBS.
South Africa	Lilian *et al. (2010)(150)*	235 samples Whatman 903	NucliSens EasyQ PCR v.? (BioMerieux)	100 (<3months old), 100 (3-12 months old)	95.6 <3m 100 3-12m	DNA more stable than RNA. Stored at -20 detection ok until 9 months storage except low VL which were stored at -70 (Leelawiwat *et al.*)
Senegal	Kebe *et al. (2011)(148)*	149 samples Whatman 903	NucliSens EASYQ HIV v1.2(BioMerieux)	100	100	Age; 3 weeks – 24 months (Kebe *et al.*)
HIV 1 NAAT DNA & RNA USA*(153)*	Huang *et al. (2011)(153)*	45x6 sets Whatman 903	RealTime HIV-1 qualitative (Abbott)	NR	100	LoD 95% Probit was 3085 IU/ml (Huang *et al.*)
7 countries worldwide	Kerr *et al. (2009) (149)*	291 samples Whatman 903	APTIMA HIV-1 RNA Qualitative Assay (Gen-Probe)	99.2	100	HIV 1. LoD 20-200 copies/ml, lower in DBS due to contribution of cellular RNA. 1 false pos was stored for 4years and had low initial VL (Kerr *et al.*)
Tanzania	Lofgren *et al. (2009)(144)*	176 samples Whatman 903	RealTime HIV-1 quantitative (Abbott)	100	99	Results at a threshold of >1000 copies/ml (100% for sens & spec at >10,000)

Disease, Assay type & country	Author	Number of samples & filter paper type	Test	Sensitivity %	Specificity %	Notes %
USA	Nugent *et al. (2009)(151)*	spiked samples only Whatman 903	APTIMA HIV-1 RNA Qualitative Assay(Gen Probe)	100	NR	For RNA vs DNA at >1000 copies/ml (86 & 100% at >10,000)
South Africa	Stevens *et al. (2008)(147)*	800 samples Whatman 903	Cobas AmpliPrep/ TaqMan HIV-1 Qual. (Roche)	99.7	100	LoD: 1x6mm punch = 10,217 copies/ml. 1 spot (13mm) = 2384 copies/ml
South Africa	Stevens *et al. (2009)(152)*	494 samples Whatman 903	APTIMA HIV-1 RNA Qualitative Assay (Gen-Probe)	100	99.7	
HIV-1 confirmation India	Solomon *et al. (2002)(137)*	89 samples Whatman 903	Western Blot (Biorad)	92	100	
HTLV 1 Serology Japan, UK, South Africa	Parker *et al. (1995)(157)*	26 samples Whatman 903	Latex Agglutination Test, Serodia HTLV1, (Fujirebio)	100	100	Based on a reference panel of samples from known positives.
HTLV 1 NAAT Japan	Noda *et al. (1993)(156)*	53 samples Whatman 903	In-house PCR	100	100	DNA remained detectable after 7 weeks storage at room temperature.

10.2.4 *Summary of studies evaluating DBS for Hepatitis viruses*

Disease, assay type, Country	Author	Number of samples & filter paper type	Test	Sensitivity %	Specificity%	Notes
HCV serology Australia*(103)*	Croom *et al. (2006)(103)*	183 samples Whatman 903	Monolisa anti-HCV PLUS Version 2 EIA (Bio-Rad)	100	100	Choice of elution buffer influences OD value
UK	Judd *et al. (2003)(158)*	643 samples Whatman 903	Ortho HCV 3.0 (Ortho)	99.2	100	Adjusting cut-off can improve sensitivity
France	Tuaillon *et al. (2010)(159)*	200 samples Whatman 903	Ortho HCV 3.0 (Ortho)	99	98	
HBV (HBsAg) Gambia*(160)*	Mendy *et al. (2004)(160)*	209 samples W. BFC180	Determine HBsAg (Abbott)	96	100	Also applied Alpha-foeto protein tests on DBS samples for hepatocellular carcinoma
Brazil	Villar *et al. (2011)(447)*	133 samples Whatman 903	ETI-MAK4 (DiaSorin)	97.6	96.7	
HBV (anti-HBc) Brazil	Villar *et al. (2011)(447)*	155 samples Whatman 903	ETI-AB-COREK PLUS (DiaSorin)	90.5	92.6	Storage at -20 gave lowest OD variation, suggesting best storage condition
HBV (anti-HBs) Brazil	Villar *et al. (2011)(447)*	134 samples Whatman 903	ETI-AB-AUK-3 (DiaSorin)	78	97.3	
HCV NAAT Italy	Solmone *et al. (2002)(163)*	53 samples	Versant HCV TMA (Bayer)	100	>95	

Disease, assay type, Country	Author	Number of samples & filter paper type	Test	Sensitivity %	Specificity%	Notes
France	Tuaillon *et al. (2010)(159)*	200 samples Whatman 903	Cobas Taqman HCV (Roche)	97	NR	HCV RNA is susceptible to degradation, store DBS at -20° C
HEV NAAT	Merens *et al. (2009)(448)*	89 samples Whatman	In-house (Enouf 06)	84	88	Multiple tests are necessary for HEV to obtain high sensitivity
Sudan	Merens *et al. (2009)(448)*	92 samples Isocode stix	In-house (Enouf 06)	93	96	HEV viremia detectable for >39 days after onset of jaundice

NR= not reported

10.2.5 Summary of studies evaluating DBS for other viruses

Disease, assay type Country	Author	Number of samples & filter paper type	Test	Sensitivity %	Specificity %	Notes
CMV serology USA	Dowd *et al. (2011) (106)*	75 samples Whatman 903	CMV IgG (Diamedix)	93	94	
EBV serology Indonesia	Fachiroh *et al. (2008) (179)*	140 samples Whatman 903	In-house IgA ELISA (fachiroh 06)	80 96*	100 93.6*	* performance on venous blood instead of capillary blood
	Fachiroh *et al. (2008) (179)*	140 samples W.No.3	In-house IgA ELISA (fachiroh 06)	75 89*	97 97*	* performance on venous blood instead of capillary blood
HSV erology USA	Hogrefe *et al. (2002) (449)*	22 samples W. No.3	Herpeselect (Focus)	NR	NR	
Measles serology Australia*(450)*	Riddell *et al. (2002) (450)*	216 samples Whatman 903	Enzygnost IgM (Dade-Behring)	90.2	98.8	
Australia	Riddell *et al. (2003) (178)*	98 samples Whatman 903	Enzygnost IgG (Dade-Behring)	96.2	92	DBS OD values were adjusted (OD *1.28) to correlate with matching serum samples
Uganda	Uzicanin *et al. (2011) (177)*	588 samples Whatman 903	Enzygnost IgM (Dade-Behring)	98.7	88.9	2-5 weeks after rash, collected samples were 100% sensitive and specific

Disease, assay type Country	Author	Number of samples & filter paper type	Test	Sensitivity %	Specificity %	Notes
Rubella serology Australia*(451)*	Karapanagiotidis *et al. (2005)(451)*	88 samples Whatman 903	Enzygnost IgM (Dade-Behring)	96	100	DBS can be shipped internationally
Peru	Helfand *et al. (2001) (452)*	87 samples Whatman 903	Captia IgM (Sanofi)	NR	NR	94% concordance (4 DBS and 3 serum indeterminate results)
			Wampole IgG (Alere)	NR	NR	93% concordance (2 DBS and 4 serum indeterminate results)
Peru	Helfand *et al. (2007) (452)*	273 samples Whatman 903	Enzygnost IgM (Dade-Behring)	98	97	
			Enzygnost IgG (Dade-Behring)	99	98	
UK	Hardelid *et al. (2008) (453)*	73 samples Whatman 903	Enzygnost IgG (Dade-Behring) IgG ELISA (Diesse)	NR	NR	
CMV NAAT Italy	Binda *et al. (2004) (180)*	195 samples Whatman 903	CMV Early Oligo Mix (Bioline- Amplimedical)	100	100	limit of detection: 4000 copies/mL whole blood

10.2.6 *Summary of key studies evaluating the diagnosis of infectious diseases on filter paper using samples other than whole blood.*

Sample Type	Notes	Reference
Bone Marrow		
Visceral Leishmaniasis PCR	PCR positive in 34 of 35 dried bone marrow samples from patients clinically suspected of having visceral leishmaniasis.	Alam *et al.* (2009) (251)
Serum		
Hepatitis A IgM, IgG & PCR	Hepatitis A IgM & IgG ELISA was both 100% sensitive and specific compared to serum. HIV ELISA was able to detect infections of less than 6 months duration with a sensitivity of 83%.	Desbois *et al.* (2009)(244) Abe *et al.* (1998) (246) Barin *et al.* (2005) (454)
Hepatitis C PCR HIV ELISA	Hepatitis A PCR was 92.3% sensitive and 100% specific and Hepatitis C PCR 100% sensitivity and specific, though with a very small sample size. Both Hepatitis A and C showed a 10-fold drop in viral load after 4 weeks storage at room temperature.	
Plasma		
HIV Quantitative PCR	Good correlation was observed in all 3 studies, between whole plasma and dried plasma spots and also between DBS and dried plasma. HIV RNA was more stable stored on paper at room temperature than as whole liquid plasma and was stable for more than 1 year.	Ayele *et al.* (2007) (247) Cassol *et al.* (1997)(248) Brambilla *et al.* (2003)(249)
Buffy coat		
HIV Proviral DNA PCR	100% concordance of proviral DNA extracted from dried buffy coat spots with DNA extracted from whole blood.	Rossi de Gasperis *et al.* (2010)(250)

Sample Type	Notes	Reference
Skin lesion samples		
Cutaneous Leishmaniasis PCR on dried skin exudates	Sensitivity ranged from 92.3 to 100% and specificity up to 100% using PCR directly on a lesion sample as the reference sample. Filter paper comparable to PCR on tissue and superior to microscopy and culture. Leishmania speciation also successfully performed.	Fata *et al.* (2009) (253) Boggild *et al.* (2010)(252) Kato *et al.* (2010) (455) Aye *et al.* (2011) (254)
Leprosy PCR on skin slit smears	116 of 192 multibacillary leprosy patients positive on FTA Elute paper. Equivalent to standard method of storage in 70% ethanol.	
Breast milk		
HIV Quantitative PCR	No statistical difference between viral load in dried breast milk and breast milk in lysis buffer (gold standard).	Ayele *et al.* (2007) (247)
Sputum		
HIV PCR	Low sensitivity for the detection of HIV RNA in saliva compared with whole blood.	Kakizawa *et al.* (1996)(456) Chibo *et al.* (2005)(256)
Measles PCR	Dried saliva less sensitive than whole saliva & nose/throat swab for measles by PCR. 67% of serologically confirmed cases positive by PCR.	Guio *et al.* (2006) (457) Mharakhurwa *et al.* (2006)(257)
HHV 6 & 7 PCR	Sensitivity and specificity for HHV6 & 7 were comparable with whole saliva samples.	Zerr *et al.* (2000) (458)
Tuberculosis PCR	*M. tuberculosis* PCR sensitivity 82%, specificity 96% - greater than microscopy. DNA detected even after 6 months storage at room temperature. Thickest parts of purulent sputum must be applied to paper.	Nuchprayoon *et al.* (2007)(258)
Malaria (P. falciparum) PCR	Malaria DNA detectable in saliva and urine. Sensitivity poor when compared with microscopy. Refinements to methods required.	
Pneumocystis jirovecii PCR	Induced sputum and brochoalveolar lavage dried on paper had similar sensitivity and specificity of 67 & 90-91% - compared with direct PCR.	

Sample Type	Notes	Reference
Cervical sample		
HPV PCR	Two studies reported excellent concordance of 94-100% between filter paper and cervical smear or cytobrush samples(259, 260). Banura *et al.* found low agreement with a kappa of only 0.18. DNA is stable for 1 year at room temperature.	Gustavsson *et al.* (2009)(259) Kailash *et al.* (2002)(260) Banura *et al.* (2008)(261)
Stool		
Vibrio cholera culture	No significant difference between filter paper and standard transport medium for culture of *Vibrio cholera.*	Page *et al.* (2011) (263)
Enterocytozoon bieneusi PCR Adenovirus 40 & 41 PCR Norovirus PCR Rotavirus PCR	Samples viable after 14 days storage when kept moist. Viral enteric pathogens are all readily stored and identified by PCR from filter paper after up to 4 months.	Carnevale *et al.* (2000)(459) Zlateva *et al.* (2005)(266) Wollants *et al.* (2004)(264) Rahman *et al.* (2004)(265)
Urine		
CMV PCR	90% concordance with direct urine DNA extraction and PCR	Nozawa *et al.* (2007)(267)
Cerebrospinal fluid		
Streptococcus pneumoniae & Haemophilus influenzae PCR	Sensitivity and specificity for *Streptococcus pneumoniae* and *Haemophilus influenzae* were 92 & 70%, and 99 % 100% respectively.	Peltola *et al.* (2010)(262)
Neurocysticercosis ELISA	There was good correlation in genome counts between liquid and dried CSF. High specificity of >90% but low sensitivities of 52-63% depending on the type of paper used.	Fleury *et al.* (2001)(227)

10.2.7 STARD CHECKLIST

STARD CHECKLIST FOR REPORTING OF STUDIES OF DIAGNOSTIC ACCURACY

ADJUSTED FOR DRIED BLOOD SPOT EVALUATION STUDIES

Amendments highlighted in blue

(version August 2012)

Section and Topic	Item #		On page #
TITLE/ABSTRACT/ KEYWORDS	1	Identify the article as a study of diagnostic accuracy (recommend MeSH heading 'sensitivity and specificity').	
	2	Make use of terminology: i.e. Dried Blood Spots, Dried Serum Spots, Dried Urine Spots, Dried Fluid Spots, etc.	
INTRODUCTION	3	State the research questions or study aims, such as estimating diagnostic accuracy or comparing accuracy between sample types or across participant groups.	
METHODS			
Participants	4	The study population: The inclusion and exclusion criteria, setting and locations where data were collected.	
	5	Participant recruitment: Was recruitment based on presenting symptoms, results from previous tests, or the fact that the participants had received the index tests or the reference standard?	
	6	Participant sampling: Was the study population a consecutive series of participants defined by the selection criteria in item 3 and 4? If not, specify how participants were further selected.	
	7	Data collection: Was data collection planned before the index test and reference standard were performed (prospective study) or after (retrospective study)?	
	8	Sample collection: State which filter paper was used, which and how fluids were obtained and spotted onto filter paper, and the drying period before storage	
Test methods	9	The reference standard and its collection, storage, and transportation details. If reference sample was not performed with the same tests or manner as the index sample, provide detailed rationale	
	10	The index sample and its collection, storage, and transportation details. Provide detailed rationale for discordances in methods between index and reference test	
	11	Sample processing: state the time and storage conditions (humidity control and temperature) at the field, during transportation, and at the laboratory, preferably in a tabled manner.	
	12	Punching method with reference to source or manufacturer, and cleaning procedure, if used.	
	13	Technical specifications of material and methods involved including how and when measurements were taken, and/or cite references for index tests and reference standard.	
	14	Definition of and rationale for the units, cut-offs and/or categories of the results of the index sample and the reference standard.	
	15	For quantitative or numerical test results, indicate the calculation methods and rationale of the index and reference standard	
	16	The number, training and expertise of the persons executing and reading the index sample and the reference standard.	
	17	Whether or not the readers of the index tests and reference standard were blind (masked) to the results of the other test and describe any other clinical information available to the readers.	
Statistical methods	18	Methods for calculating or comparing measures of diagnostic accuracy and bias (e.g. Bland Altman), and the statistical methods used to quantify uncertainty (e.g. 95% confidence intervals).	
	19	Methods for calculating test reproducibility, lower limit of detection, if done.	
	20	For quantitative test outcomes, the mean and range of results for index and reference test.	

RESULTS		
Participants	21	When study was performed, including beginning and end dates of recruitment.
	22	Clinical and demographic characteristics of the study population (at least information on age, gender, spectrum of presenting symptoms).
	23	The number of participants satisfying the criteria for inclusion who did or did not undergo the index tests and/or the reference standard; describe why participants failed to undergo either test (a flow diagram is strongly recommended).
Test results	24	Time-interval between the index tests and the reference standard, and any treatment administered in between.
	25	Distribution of severity of disease (define criteria) in those with the target condition; other diagnoses in participants without the target condition.
	26	A cross tabulation of the results of the index tests (including indeterminate and missing results) by the results of the reference standard; for continuous results, the distribution of the test results by the results of the reference standard.
	27	Any adverse events from performing the index tests or the reference standard.
Estimates	28	Estimates of diagnostic accuracy and measures of statistical uncertainty (e.g. 95% confidence intervals).
	29	For quantitative test outcomes, Estimates of diagnostic accuracy and measures of statistical uncertainty (e.g. 95% confidence intervals) by quantitative grouped ranges (e.g. 1,000-5,000 copies/mL).
	30	How indeterminate results, missing data and outliers of the index tests were handled.
	31	Estimates of variability of diagnostic accuracy between subgroups of participants, readers or centers, if done.
	32	Estimates of test reproducibility, if done.
DISCUSSION	33	Discuss the clinical applicability of the study findings.

10.2.8 REFERENCES OF EXCLUDED IN-HOUSE ASSAYS

HIV

Comeau(460) De Crignis(283) luo(461)Mehta(462)Ou (463)Panteleeff(464) Nyambi(465)Jacob(466) Chohan(467) Walter(468) Zhang(469)Yourno(470, 471) Barin(454)Beck(472) Bellisario(473)Cassol(474, 475) Lindhardt(476) Newell(477)Sriwanthana(478)

HEPATITIS B & C

Parker(479) De Crignis(283) Gupta(480) Lira(481)

CMV

Soetens(482) Scanga(483) Vauloup-Fellous(484) Yamamoto(485)Atkinson(486) Barbi(487, 488) Boppana(489)

MEASLES

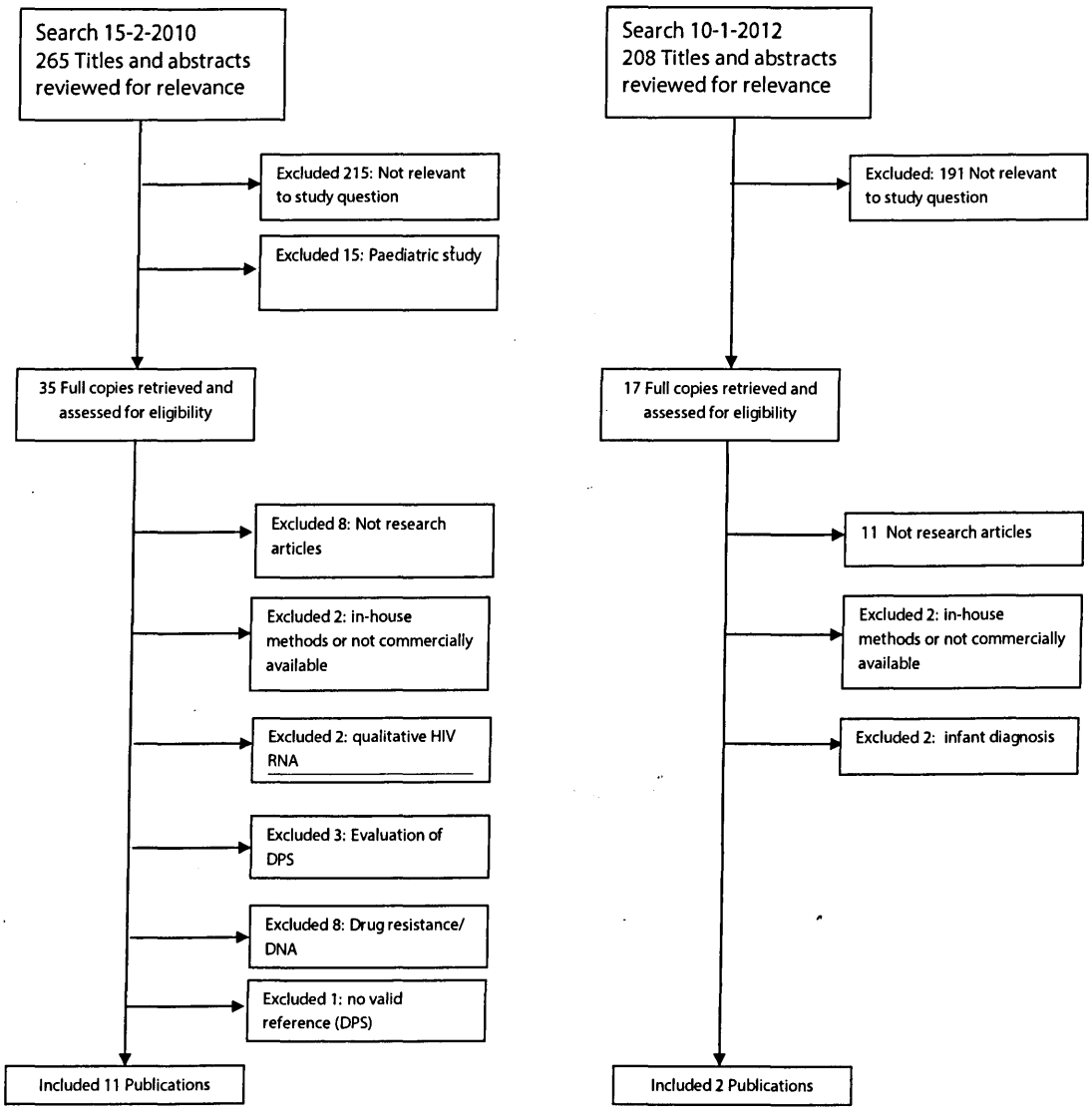
Condorelli(490)De Swart(491) El Mubarak(492)

RUBELLA

Punnarugsa(493)

10.3 ANNEXES TO CHAPTER 3

10.3.1 INCLUSION AND EXCLUSION ALGORITHM



10.3.2 *Search strategy protocol*

Systematic review of HIV DBS: Search strategy protocol

Databases

MEDLINE: All aspects of clinical medicine, biomedicine, nursing, dentistry, allied health, health policy, genetics etc. Emphasis on English-language source, quite biased towards journals published in N. America. Includes journal articles from 1950s onwards and is updated daily.

EMBASE: All aspects of clinical medicine, biomedicine, nursing, dentistry, allied health, health policy, genetics etc. Quite biased towards information in journals published in Europe. Particularly strong on pharmaceutical information. Includes journals from 1947 onwards and is updated weekly.

Search Terms

Search Embase:

("1998/01/01"[Publication Date] : "3000"[Publication Date]) AND ((((dried[tw] OR dry[tw]) AND (spot[tw] OR spots[tw]) AND blood[tw]) OR dbs[tw] OR filter paper[tw] OR filter papers[tw] OR guthrie card*[tw] OR 903 paper[tw]) AND (hiv*[tw] OR human immunodeficiency virus*[tw] OR human immune deficiency virus*[tw]) AND (DNA* OR RNA OR infant OR neonat*)

Search medline:

- ((dried or dry) and blood and spot*)
- (dbs or "filter paper*" or "guthrie card*" or "903 paper")
- (hiv* OR "human immunodeficiency virus*" OR "human immune deficiency virus*" OR HIV-1)
- (DNA[tw] OR RNA OR infant OR neonate OR neonatal OR infants*)
- ("early infant diagnosis" OR EID)
- limit to yr="1998 -Current"
- 1 OR 2 AND 3 AND (4 OR 5)

Date

1998 to present

Language

English

Publication type/status

Published works in peer reviewed journals.

Study selection

Stage 1: Screening of titles/ abstracts against inclusion criteria.

Titles and abstracts, where available, will be screened and either accepted, rejected as not relevant, or rejected due to failure to meet inclusion criteria (if so, the reason will be specified).

Stage 2: Full papers obtained and assessed against inclusion criteria. Papers will be either accepted or rejected due to failure to meet inclusion criteria and the reason will be specified.

Full papers will be independently assessed by TWO members of the review team and results will be cross-checked and combined.

Inclusion Criteria

- Evaluation or comparison of performance of commercially available DNA/RNA assays with DBS and reference sampling methods
- Evaluations based on human clinical or reference materials

Exclusion criteria

- Not an evaluation study or not having a correct reference sample or reference method
- In-house developed assays or a no longer commercially available assay
- Studies with other primary aims other than evaluation of DBS for early infant diagnosis with reference samples *
- Studies related to drug resistance screening, genotyping, sequencing, other "non-diagnostics" evaluation studies
- Studies with a population that does not include newborns or infants
- Study written in a language which is not English

Data Extraction

General Information
Date of data extraction
Identification features of the study:
Record number
Author
Article title
Citation
Study Characteristics
Aim/ objectives of the study
Filter paper type
Participant/ Sample Characteristics
Characteristics of population from which samples were drawn:
Age
Sex
Number of samples
Sample country/ region of origin
Sample type (finger prick/ EDTA/..)
DBS storage:
- At research site
- At laboratory

Use of desiccants
Type of reference sample
DBS quality check performed?
Technology
Name and manufacturer of assays under evaluation
Extraction method
Extraction volume/adjustments
Extraction kit/ method used
Detection method
If quantitative; adjusted for DBS sample input?
Outcome data/ results
Unit of assessment/ analysis

Outcomes:
NA:
Mean pathogen load + standard deviation
Range of pathogen load
Correlation (r)
Bias (mean difference)
Sensitivity, cut-off
Specificity, cut-off
% CV index test, reference test
Serology:
Sensitivity, cut-off
Specificity, cut-off
Cut-off adjusted for DBS?
Titre lower limit of detection
Variability, cut-off, replicates
For each pre-specified outcome:
Reported (Y/N)
Definition used in study
Additional outcomes reported
Details of any additional relevant outcomes reported

Quality Assessment

Title/ abstract	Is the article easily identified as a study of test evaluation?
Introduction	Does it clearly state the research question and study aims?
Methods	Do the authors clearly describe the study design? Are study inclusion and exclusion criteria provided? Were steps taken to introduce blinding and random allocation where possible and appropriate?
Participant/ Sample Characteristics	Is the population from which the samples were drawn described? Is the country/ region of sample origin detailed? Were the samples collected prospectively? Do the authors describe how the samples were acquired, stored and prepared? Was the choice of anticoagulant appropriate for the technology?
Test methods	Were staff trained in the use of the technology prior to performing the index and reference tests? Was the reference standard explained in sufficient detail to be reproduced? Is the index test explained in sufficient detail to be reproduced? Did the authors report the number of technicians reading the index/ reference tests? Was a single sample divided and tested by each technology included in the study or were a different set of samples used for each test?
Statistical methods	Were the methods used reported in detail? Were the methods used appropriate?
Results	
Participants	Were the demographic characteristics of the population described?
Test results	Was the distribution/ range of viral load of the sampled population reported? How were the data presented? Was subgroup analysis performed for different HIV-1 subtypes?
Discussion	Was the clinical relevance of the study findings discussed?

10.3.3 Summary of studies that evaluated the use of DBS for HIV VL and EID

Author	sample origin	ART +/-	# samples	Spot µl	# spots used	DBS elution volume	Elution process	Extraction method	Detection Platform
HIV viral load									
Andreotti et al	Malawi	neg	102	75	1	2 ml	Overnight, room T	NucliSens miniMAG	COBAS TAQMAN RT analyzer
Arredondo et al	Spain	70% neg	154	50	2	2ml	120 min, room T	M2000sp	Abbott RealTIME HIV-1
Garrido et al	Spain	-	103	50	1	2 ml	2 hours, gentle rotation	Manual NucliSens kit	NucliSens easyQ v1.1
Garrido et al	Spain	-	103	50	1	2 ml	2 hours, gentle rotation	M2000sp	M2000rt
Ikomey et al	Cameroon	-	60	50	-	0.9ml RBC lysis	10 min	-	Amplicor Monitor v1.5
Kane et al	Senegal	-	33	50	2	2 ml	30 min, room T	NucliSens MiniMAG	NucliSens easyQ v1.2
Leelawiwat et al	Thailand	pos	56	50	1	0.9ml	120 min, 25°C	Manual NucliSens kit	Amplicor Monitor v1.5
Lofgren et al	Tanzania	pos	137	50	2	1.7ml	2 hours, room T	M2000sp	M2000rt
Marconi et al	Italy	-	168	50	2	2 ml	2 hours, room T	M2000sp	M2000rt
Mbida et al	Cameroon	pos	41	50	2	1.7 ml	2 hours	M2000sp	M2000rt
Pirillo et al	France	-	98	50	1	1.25 ml	30 min, Room T	VERSANT preparation module	Versant HIV-1 kPCR
v Deursen et al	The Netherlands	pos	83	50	2	2 ml	30 min,	NucliSens easy MAG	NucliSens easyQ v2.0

Author	sample origin	ART +/-	# samples	Spot µl	# spots used	DBS elution volume	Elution process	Extraction method	Detection Platform
Rottinghaus et al	Nigeria	neg	173	100	1	2 ml	30 min, Room T	NucliSens easy MAG	NucliSens easyQ v1.1
Vidya et al	India	-	100	50	2	1.75 ml	2 hours, 56°C	M2000sp	M2000rt
Early infant diagnosis									
Anitha et al	India	-	766	-	1	100 ul	3 hours, 56°C, 10 min 100°C	Chelex-100 resin	Amplicor 1.5
Nsojo et al	Tanzania	-	325	20	1	200ul	15 min 60°C, 100°C	-	Amplicor 1.5
Sherman et al	South Africa	-	280	20	1	200ul	15 min 60°C, 100°C	-	Amplicor 1.5
Stevens et al	South Africa	-	800	70	7* 3.2mm	200ul	15 min 60°C, 100°C	-	Amplicor 1.5
Stevens et al	South Africa	-	800	70	1	1100ul	10 min, 56°C	Cobas Ampliprep	Taqman

10.4 Annexes to Chapter 4

10.4.1 Manuscript

The accuracy and utility of syphilis point of care tests and laboratory assays in Tanzania

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[3] National institute for Medical Research, NIMR Mwanza, Tanzania
[4] Mwanza intervention Trials Unit, Mwanza, Tanzania

Abstract

The availability of rapid and sensitive methods to diagnose syphilis facilitates screening of pregnant women, which is one of the most cost-effective health interventions available. We have evaluated four methods in Tanzania : the *Treponema Pallidum* Particle Agglutination assay (TPPA), the *Treponema Pallidum* Haemagglutination Assay (TPHA), an Enzyme Immuno Assay (EIA), and a point of care test (POCT) The POCT was performed in the clinic on whole blood, while the laboratory assays were performed on plasma at the laboratory

In total, 2459 samples were tested and gave a TPPA seropositivity of 17.3%. With TPPA as reference assay, the sensitivity and specificity of TPHA was 87.9% and 97.4%, of EIA was 95.3% and 97.8%, and of the POCT was 59.6% and 99.4% respectively. The sensitivity of the POCT to detect active syphilis cases (TPPA positive and Rapid Plasma Reagin titre ≥1/8) was 82%.

The low sensitivity of the POC test is critical and should be further assessed under routine clinical use in African developing countries. Even though the sensitivity of the POCT is low, the sensitivity of detecting active syphilis cases and large increase in accessibility of diagnostic tests makes the POCT the preferred choice for remote clinics in Tanzania.

Introduction

The prevalence of syphilis is high among pregnant women attending antenatal clinics in sub-Saharan Africa [1]. During pregnancy, syphilis can have devastating effects on the developing foetus and is a major cause of stillbirths and neonatal deaths in Africa [2-4]. Screening and treatment of pregnant women with a single dose of benzathine penicillin before the third trimester could prevent up to 500,000 stillbirths and neonatal deaths annually, in Africa alone [5].

Serological assays are important for the diagnosis of syphilis. Laboratory based assays such as the *Treponema Pallidum* Particle Agglutination (TPPA), *Treponema Pallidum* Haemagglutination (TPHA), and Rapid plasma Reagin (RPR) are widely used. As the agglutination is interpreted by a technician, the test result is subjective. By the introduction of Enzyme Immunoassays (EIA) with a plate reader, test results could be read objectively and large quantities of samples could be processed. EIAs are suggested to be more robust and more sensitive than TPPA, but are also more expensive [6, 7].

These laboratory based assays are not available in rural health facilities in Africa. POCTs are easy to perform, require only a finger prick drop of blood, and do not require refrigeration; they could enable same day testing and treatment for syphilis at any health facility

Comparative evaluations of these TPPA, TPHA, EIA and POCT assays have not been previously performed in Sub Saharan Africa [8]. Considering the major burden of syphilis in Sub Saharan Africa, this study was performed in Tanzania to evaluate the POCT against 3 laboratory assays. Besides the performance of a test, kit costs, necessary equipment, cold chain requirements, and complexity of executing tests are essential for evidence based decision making and were included in the discussion. This study is performed to aid decision makers in developing countries to select a suitable screening test for syphilis.

Methods

Samples and Field Procedures

The Kisesa open cohort is a well established ongoing community-based study in Northern Tanzania [9]. The cohort study uses regular demographic surveillance with serological surveys, providing data on HIV incidence and prevalence [10]. Subjects that accepted Voluntary Counselling and Testing (VCT) were tested for HIV and syphilis using POCT. All subjects with a positive syphilis result were given free medical treatment according to Tanzanian government guidelines, and all those positive for HIV were referred to the Tanzanian care and treatment centre. The study was approved by the Medical Research Coordinating Committee of the National Institute for Medical Research in Tanzania and the Ethical Committee of the London School of Hygiene and Tropical Medicine.

Whole blood was collected into heparinised tubes from consenting subjects, by the VCT nurse, and transported to the National institute for Medical research (NIMR) laboratory in Mwanza. Within 24 hours the blood was centrifuged and stored at -200C. Samples were collected from April 2010 until September 2010 and were tested until March 2011. Samples were bar-coded to ensure anonymous testing. Double data entry was used to enter the results. Results were entered automatically (EIA) or manually into the Laboratory Information Management System (LIMS).

Point-of-care test

The SD bioline syphilis 3.0 POC tests (Standard Diagnostics, Kyong gi-do, Korea) were performed by trained and experienced clinicians, with whole blood samples collected by venous puncture. A timer assured 15 minutes waiting time were applied before reading the test results. The manufacturer's procedures were followed.

TPPA & TPHA

A total of 2459 plasma aliquots were raised to room temperature and tested by use of TPPA (Fujirebio, Tokio, Japan) and TPHA (newlab21, UK). Both tests were performed according to the manufacturer's instructions. TPPA and

TPHA results were read by two trained and experienced technicians. The reading of the TPPA and TPHA occurred while masked to results of other tests. Discordant results between the two technicians were discussed and one outcome was agreed by consensus. Results were deemed indeterminate for biologically reactive samples or when a conclusive outcome could not be obtained due to difficulty in interpretation or lack of technician agreement.

Enzyme Immuno Assay

The Syphilis Enzyme Immuno Assay (EIA)(Lab21 healthcare, Kentford, UK) was performed on 1272 samples (50%). The EIA was included halfway during the course of the study. The EIA was carried out according to manufacturer's instructions and read by Optical Density (OD) 450/620nm by automated reader (DTX 800, Beckman Coulter, USA) which calculated the cut-off limit according to the instruction manual. The results were then entered directly into the Laboratory Information Management System (LIMS).

The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated according to standard calculations. The agreement between various methods was tabulated. Microsoft Excel (Microsoft Corp., USA), the statistical package Stata 11 (Stata corp LP,Texas, USA) were used for analysis of the results.

Results

In total, 2459 samples were tested with TPPA and TPHA, 2099 (85.3%) POCT were performed and 1273 (51.7%) were tested using the EIA. The syphilis seroprevalence was according to TPPA 17.1%, for TPHA 17.1%, for EIA 19.3% and 10.7% according to POCT.

The comparison between TPPA, TPHA and EIA is shown in table 1a-e. There was a 95.8% agreement between TPPA and TPHA and a 97.3% agreement between TPPA and EIA. The sensitivity and specificity of TPPA, TPHA and EIA are summarised in Table 2. Figure 1 shows the distribution of all positive samples identified by one or more of the laboratory assays.

Table 1. Summary of samples tested with TPPA, TPHA, SD bioline 3.0, and EIA.

		TPPA		
		+	-	total
TPHA	+	364	52	416
	-	50	1959	2009
	total	414	2011	2425*

1a. TPHA against TPPA
*33 indeterminate, 1 not tested for TPHA

		EIA		
		+	-	total
TPHA	+	205	25	230
	-	37	996	1033
	total	242	1021	1263*

1b. TPHA against EIA
*10 indeterminate excluded

		TPPA		
		+	-	Total
EIA	+	222	23	245
	-	11	1016	1027
	Total	233	1039	1272*

1c. EIA against TPPA
*1 not tested for TPPA

		EIA		
		+	-	Total
POC	+	114	6	120
	-	85	836	921
	Total	199	842	1041

1d. POCT against EIA

		TPPA		
		+	-	Total
POC	+	214	11	225
	-	145	1729	1874
	Total	359	1740	2099

1e. POCT against TPPA

All discordant and indeterminate result obtained by any of the three laboratory methods were retested by TPPA, TPHA and EIA. When retesting all discordant results, 24 out of 50 TPPA positive discordant samples became negative, and 11 out of 105 TPPA negative discordant samples became positive. For TPHA, 41 initially positive samples became negative when retested and 11 initially negative discordant results became positive. Although EIA was performed on fewer samples than TPPA and TPHA, only five samples changed outcome when retested (5 initially positive samples became negative out of 41 discordant results).

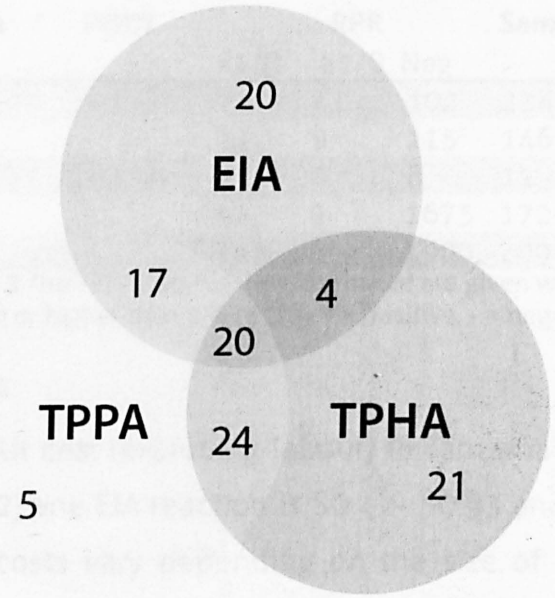

Figure1. Distribution of positive samples among the three assays. Numbers represents positive samples detected by three assays (number is given in 3 circles), or by two assays (overlapped by two circles) or by one assay (given as number below the assay name).

	TPPA				
	Sensitivity (95%CI)	Specificity (95% CI)	PPV	NPV	K
TPHA	87.9% (84.4 -90.7%)	97.4% (96.6-98.0%)	87.5%	97.5%	85.2%
EIA	95.3% (91.8-97.3%)	97.8% (96.7-98.5%)	90.6%	98.9%	91.2%
POCT	59.60% (54.5-64.6%)	99.40% (98.9-99.7%)	90.6%	98.9%	91.2%

Table 2. The TPHA and EIA performance are given against the TPPA as reference method. PPV= positive predictive value, NPV= negative predictive value, K= kappa correlation value, 95% CI = 95% confidence interval.

Point- of- care test

The performance of the POCT with TPPA as reference is shown in table 1d. To evaluate the sensitivity of detecting active syphilis cases, RPR was performed (Table 3). Of the 146 false negatives with TPPA as reference, 115 (79%) were RPR negative and 31 samples were positive. Based on the RPR interpretation of the 145 false negatives, nine active syphilis cases were missed when using POCT instead of TPPA. Based on 2099 samples, 50 had an active syphilis infection (positive TPPA and RPR titre ≥ 1/8) of which 41 were detected by POCT, giving a sensitivity of 82% (69.2% -90.2%).

TPPA	POCT	RPR			Samples
		<1/8	≥1/8	Neg	
+	+	71	41	102	214
+	-	22	9	115	146
-	+	3	0	8	11
-	-	44	9	1675	1728
Total		140	59	1900	2099

Table 3 The TPPA and POCT performance are given with RPR results divided into titres lower (<1/8) or higher than 1/8 (≥1/8). += positive, - = negative, Neg=negative

Costs

The kit cost (excluding labour) in Tanzania of one TPHA reaction is $0.18 - $0.32, one EIA reaction is $0.42- $0.93 and one TPPA reaction costs $0.46. The costs vary depending on the size of the purchased kits. This simple calculation does not include the number of negative and positive controls necessary per run, which is higher for the EIA. The cost of the POC test was $ 1.10 per test in Tanzania.

Discussion

To our knowledge, this is the first study comparing TPPA, TPHA, POCT and EIA performed in an African setting. The TPPA, TPHA and EIA were comparable in performance, with TPHA yielding the lowest performance (88.4% sensitivity).

It is important to note that this study was performed in Tanzania and on Tanzanian samples, which potentially influence the results. African samples can contain high immunoglobulin levels due to other infections which potentially cause false positive test results [11, 12].

Although tests were performed and stored according to manufacturer's recommendations, potential environmental effects by transporting and using the kits under tropical conditions could not be completely ruled out. Therefore, performing tests in an African country relies more heavily on the robustness of the tests than in developed countries. This could potentially influence the test results.

Based on the results found in this study, it seems that both the EIA and TPPA perform well and are suitable to be used for syphilis screening in developing countries. The TPHA is less suitable due to limited reproducibility of results,

lower sensitivity compared to TPPA or the EIA, and more equivocal results. The reproducibility of the TPHA was limited due to the high number of samples that changed outcomes when retested, which could be an indication of difficulties in test interpretation or reduced reliability with low titre samples.

There were very few discordant EIA results between the first test results and when retesting discordant results. Thesamples that were discordant had very low OD measures, just above the cut-off. Therefore, EIA seems to be a more stable and reliable test, but it is debatable if the benefit outweighs the costs compared to TPPA. EIA's require a plate washer and a reader for objective interpretation of the results. Additionally, EIA's laboratory procedures require more steps and more controls per run than TPPA, which could make TPPA a preferable option for handling smaller sample volumes. When a laboratory is experienced in interpreting agglutination results, it may be preferable to continue the use of TPPA instead of switching for the more objective and more expensive EIA.

The performance of the POCT (59.6% sensitivity and 99.4% specificity) was below the manufacturer's claim (99.3% sensitivity and 99.9% specificity). This is consistent with another multi-country comparison study conducted in Africa which found a performance below the manufacturer's claims, although the sensitivity of POCT was higher than in this study (85.7%)[4]. This finding is concordant with other studies that found a reduced sensitivity when the POCT is performed on whole blood [13, 14]. Additionally, this study compared the POCT in a field setting while laboratory tests were performed on plasma.

For routine screening in developed countries, treponemal and non-treponemal tests are combined to differentiate past and current syphilis infection. The positive samples that were missed by POCT were mostly RPR negative (77%) and only 9 out of 50 active syphilis cases were missed. This finding suggests that POCT is less sensitive than laboratory tests but still capable of detecting most active syphilis cases. Even though the sensitivity is low, POCT provides additional benefits compared to laboratory based assays. POCT require no refrigeration, provide fast results, and are easy to operate, enabling health care workers without access to laboratories to offer a same

day testing and treatment service [15]. The alternative test that was used at some rural clinics in Tanzania was RPR. If a health centre has a freezer and plate rotator, RPR can be performed, although the results obtained in rural clinics in Africa have not been encouraging [13].

The acceptability of losing sensitivity while gaining accessibility of diagnostic tests is often misjudged. To emphasise the importance of accessibility of diagnostic tests, a study was conducted that evaluated the implementation of POCT in antenatal care clinics in Tanzania (Mabey, Plos one, in press). The use of POCT instead of RPR tests led to a 4 times increase of pregnant women being tested (3.561 with RPR versus 14.562 with POCT in 3 months). Because of this, 8.4 times more positive cases were detected with POCT. Although the sensitivity of POCT is low, the increased accessibility of syphilis diagnostics increased the number of women screened and adverse births effects prevented. An improved POCT would be preferable but at the moment, these POCT are an acceptable solution for remote clinics in Tanzania.

Conflict of interest

None to declare

Acknowledgements

The TPHA and EIA were kindly donated by the manufacturers for this evaluation.

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10.5 ANNEXES TO CHAPTER 5

No Annexes to Chapter 5.

10.6 ANNEXES TO CHAPTER 6

10.6.1 PROTOCOLS DEVELOPED

Quality assurance protocol for dried blood spots
Standard operating procedure 3.0

Subtitle: Laboratory protocol for TPPA testing using Serodia Fujirebio test kit
Protocol ID: 3.0
Date: March 2012
Created by: Pieter Smit, Thomas van der Vlis
Email address: Pieter.smit@lshmt.ac.uk

Purpose of protocol:

Serodia TPPA test kit from Fujirebio inc. for qualitative detection of Treponema Pallidum antibodies is used for the detection of syphilis on DBS samples. This protocol describes in detail the testing procedures when using DBS samples. The procedure is divided into two stages. The first stage explains the creation of a masterplate which needs to be incubated overnight. The second stage describes the TPPA test kit protocol using the masterplate as sample material. Test result interpretation is qualitative and described at the end of this SOP.
TPPA testing should be performed after DBS card quality evaluation (protocol ID 2.0). Printed 96 wells templates are needed to record test results. If bar-coded stickers on the DBS cards are used the printed template might be exchanged for an excel using a bar-code scanner device. The template to be printed can be found at the global health diagnostic website.

HEALTH AND SAFETY INFORMATION CAUTION:

You are working with potentially infective materials. Read the manufactures manual for safety regulation.

Procedure (1):

Materials needed:

- Specimen DBS cards + empty DBS
- Punch machine or punchers
- Disposable gloves
- Disposable tips
- Pipette's (multi-pipette)
- PBS buffer (96*100ul = 9,6ml for 1 plate)*
- 96 flat wells plate + cover
- Printed 96wells template
- Plate shaker

* see page 4 (bottom) for detailed PBS-buffer requirements.

Operations:

- Collect (sample) DBS cards
- Set up 96 wells plate (number the plate for reference)
- Leave well A1 empty for positive control
- Punch blank card at B1 for blank control
- Punch samples into the well's starting at C1 (followed by D1 etc)
- For each punch, write down card-number on printed template
- For each punch, write down what punch (number) you used in the small square (optional)
- Add 100µl of PBS buffer to each well (using multi-pipette)
- Tap the plate from the side (3x), the spots will turn
- Shake plate for ~30sec on a shaker
- Cover wells and place in a refrigerator (4 degrees Celsius) overnight to elute

This is your master plate.

Do not store the plate longer than 2 days (at 4 degrees Celsius)

Procedure (2):
Serodia TPPA test (following within 2 days after creating master plate).

Materials needed:
- (Multichannel) Pipette's
- Timer
- Gloves
- Disposable tips
- U-shaped 96 wells plate (2x per one fully filled masterplate)
- 1.5ml vial
- Pen

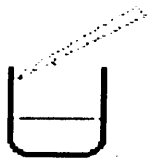
Pre-test
- Prepare reagents **30min** in advance.
- Bring TPPA test kit at room temperature.
- Bring the Master plate at room temperature.
- Every test requires 2 wells, so for a full master plate, 2 u-shaped 96wells plate are needed.

Operations
- Number the U-shaped 96 wells plate 1 and 2 if applicable and mark the plate's with numbers as on the masterplate. U-shaped 96 wells = U96W
- Pipette **25µl** of sample diluents into the whole first column (A1-H1)
- Repeat this step for column 3; 5; 7; 9 and 11 (A3-H3; A5-H5; A7-H7 etc) and go on, on the 2nd U96W plate if necessary.
- Elute **25µl** of positive control (from kit) and **190µl** sample diluents into a vial and mix thoroughly, with pipette.
- Pipette **25µl** of the positive control mix into well A1 of the U96W plate

Read the following 4 steps first before proceeding

- Pipette **25µl** of each well from the first column of the master plate into the first column of the U96W plate
- Mix specimen solution with sample diluent in U96W plate with pipette, thoroughly.
- Without changing tips, pipette **25µl** of the diluted specimen solution into the column next to it (first column A1-H1 à A2-H2)
- Repeat for the other columns of the master plate. Column two of master plate must be pipetted into column 3 of the U96W plate, column 3 of the master plate into column 5 of the U96W plate etc. *(column 7 of the master plate must be pipetted into column 1 of the second U96W plate.)*

- Pipette **25µl** unsensitized particles with dropper or multi-pipette in every **even** column (A2-H2; A4-H4..etc)
- Pipette **25µl** sensitized particles in each well of every first (**odd**) column (A1-H1; A3-H3; A5-H5 etc)
- Cover the U96W plate's and shake the plate's with shaker for about 20sec
- Incubate at room temperature for at least **2 hours**.
- **Keep the plate free from vibrations**



**if you are using the multichannel pipette instead of the provided droppers to add the unsensitized and sensitized particles, make sure that you place the pipette tips high on the wells to minimize contamination (see figure below). This is only of relevance when you are not changing tips.*

Test interpretation:

- After incubation period, make a photo of the plate's
- Write down the results on the printed template (P = positive N = negative I = Indetermined)
- Every odd column of the U96W plate corresponds with consecutive columns on the printed template
  - Fill in results Column 1 of 1st U96W on column 1 of the printed template
  - Fill in results Column 3 of 1st U96W on column 2 of the printed template
  - Fill in results Column 5 of 1st U96W on column 3 of the printed template
  - Fill in results Column 7 of 1st U96W on column 4 of the printed template
  - Fill in results Column 9 of 1st U96W on column 5 of the printed template
  - Fill in results Column 11 of 1st U96W on column 6 of the printed template
  - Fill in results Column 1 of 2nd U96W on column 7 of the printed template
  - Fill in results Column 3 of 2nd U96W on column 8 of the printed template
  - Fill in results Column 5 of 2nd U96W on column 9 of the printed template
  - Fill in results Column 7 of 2nd U96W on column 10 of the printed template
  - Fill in results Column 9 of 2nd U96W on column 11 of the printed template
  - Fill in results Column 11 of 2nd U96W on column 12 of the printed template

- Particles are concentrated in the shape of a button at the center of the well with a smooth (sharp) round outer margin. **Read: (-)** Non-reactive
- Particles are concentrated in the shape of a compact ring with a **very small** 'hole' in the middle and a smooth (sharp) round outer margin. **Read: (-)** Non-reactive
- Particles are concentrated in the shape of a compact ring with a 'hole' the middle and a smooth round outer margin. **Read: (+/-)** Inconclusive

- Defined large ring with a rough multiform outer margin and peripheral agglutination. **Read: (+)** Reactive.
- Agglutinated particles spread out covering the bottom of the *Well* uniformly. Edges sometimes folded. **Read: (++)** Reactive.

Interpretation criteria:

Check if all unsensitized particles are negative

Positive: specimen is (-) with unsensitized particles, but (+) with sensitized particles

Negative: (-) or (+) with unsensitized particles, if sensitized particles is (-)

If unsensitized and sensitized are both positive, the sample must be re-tested.

Test validity

The test is valid when the positive control is reactive.

Limitations of procedure

- Alterations in the physical appearance of the test kit materials may indicate instability or deterioration
- All highly sensitive assays have the potential for non-specific reactions
- Adaptations have been made to the manufacturers' protocol.
- This protocol is established and validated within our laboratory setting. A test validation within your laboratory setting is highly recommended.

PBS-buffer

Needed: PBS buffer with 0.05% tween20

Example:

80ml PBS

40ul tween

Use 'positive displacement' pipette tips to add the tween20 to the PBS. This is a very thick soap, and normal tips will not work.

Quality assurance protocol for dried blood spots
Standard operating procedure 2.0

Subtitle: Laboratory protocol for the quality evaluation of dried blood spot samples.
Protocol ID: 2.0
Date: March 2012
Created by: Pieter Smit, Thomas van der Vlis
Email address: Pieter.smit@lshmt.ac.uk

Purpose of protocol:

Dried Blood Spots (DBS) samples are used in various settings and used for different purposes throughout the world. As originally intended for neonatal screening, DBS are currently used for surveillance, diagnostics, quality control or infection monitoring.

The quality of a clinical sample is essential for good quality diagnostic testing, irrespective of which tests are applied. Based on a study for the development of a quality assurance method for rapid diagnostic tests, a protocol was developed to review the quality of DBS. As DBS are susceptible for environmental influences, test results could be influenced by the quality of the spot.

In this protocol, the quality criteria for DBS samples are incorporated into the laboratory process. The protocol makes use of the form to write down the quality of the DBS samples. This form is called: DBS quality assessment template.docx and can be found at the global health diagnostic website as well.

HEALTH AND SAFETY INFORMATION CAUTION:

You are working with potentially infective materials. Read the manufactures manual for safety regulation.

Procedure:

Materials needed:

- Desiccants (preferably color changing)
- Zipper lock bags (Large & small)
- Notation stickers (optional)
- QA forms
- Disposable gloves
- substance mask (optional)

Operations:

One form can contain 30 entries (1 form double sided printing).

- Note date and sample location on the form.
- Note if there were desiccants in the bags and if new ones were necessary on the form.
- Assess (see below for details) the individual DBS cards and fill in the form.
- When the form is completed, place the cards in a 'small' zipper-lock bag with enough new desiccants (about 1-3 desiccants).
- Note the "bag-number" (starting from 1) on the form and on the bag (use notation stickers).
- Press as much air out of the bags as possible and seal bag.
- Place 5 smaller zipper-lock bags in one large one and note bag-numbers.
- For long term storage (over 2 weeks), store at minus 4 degrees Celsius. For short term storage you may place the bags in a refrigerator (4-6 degrees Celsius)

Assessment of DBS cards:

Punch 1-5:
Fill in code 1 through 9 for every spot. The most left spot represents spot 1. The codes representations are given below in the visual examples. As most examples are easily recognized, 2 codes are highlighted below:

Code 3: Contamination (mold) can occur. Discoloration and other forms of contamination should also be marked with code 3

Code 5: Layered spots occur more commonly. Layered spots will be fairly easy to recognize because of darker blood spots above lighter ones. Spots with different colors and scratches can also be marked with code 5

(general) card impression:
Assess the card itself. Is it severely damaged or very dirty on the in- and/or outside? Does it look like the sample has been very wet? If this is so, a negative assessment should be given. The 'card impression' does not judge individual spots, only the card itself.

Humidity:
Humidity can have serious influences on test results so registration is important. Assess the color of the desiccants on arrival. If they are green the cards get code 2 (if the cards are dry). If the card appears to be wet, mark with code 4. If it looks like the card has been (very) wet in the past, mark with code 5 (e.g. wrinkled and frayed)

Tampered sticker:
If stickers are used on the cards for registration, it can be useful to look if the stickers have been tampered with since this could lead to errors in correct registration. If stickers have been removed and re-pasted this can often be seen quite easily. Use code 2 if the sticker has been tampered with and code 3 if you are not sure.

Usable?:
Depending on the set requirements, the DBS cards can in theory always be used if they contain enough blood. Sometimes, however, it might be necessary to discard a DBS (e.g. high level of contamination with mold).

Note:
Space to record extra information. Sometimes the codes on the form will not be sufficient to describe a DBS card properly. This area can be used to supplement extra information (e.g. bugs on the cards).

DBS assessment results
When a DBS card contains good quality spots, the test procedure can be initiated. If the card contains none acceptable spots, this sample should be recorded as "not usable". If this is the case, it is recommendable to exclude the sample for further analysis.

Recording of the results
We recommend entering the quality assessment data into a database. To be able to relate the quality of the DBS samples with test outcome, the data should be linked with test results. It greatly depends on the setting which databases are available or which can be used. If no databases are suitable, Epi-info could be a suitable alternative. Please find more details about Epi- info here: (http://wwwn.cdc.gov/epiinfo/)

Legend as on QA form;

- Sticker# = card/stickernumber
- Pun1-5 = spot number from left to right

Values 1-7 in consecutive order (see below for invalid samples):

1. good spot
2. insufficient quantity
3. diluted/discolored or contaminated
4. exhibits serum rings
5. clotted or layered
6. no spotting
7. Un-determined

- CI= (general) card impression

Values 1-3 in consecutive order:

1. Good
2. Bad
3. Doubtful
9. Un-determined

- Hum = Humidity

Values 1-5 in consecutive order:

1. dry with yellow desiccants*
2. dry with green desiccants
3. Moist
4. (very) wet
5. signs of past humidity
9. Un-determined

**If no color changing desiccants were used and the card is dry use code 1.*

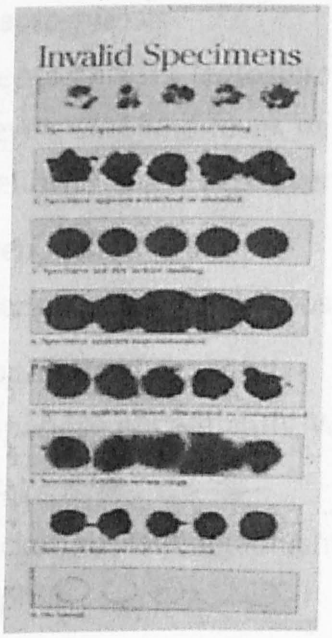
- Tamp. Stick = Tampered sticker (has the sticker been tampered with?)

Values 1-3

1. No, It looks good.
2. Yes, it looks like it has been removed and re-pasted.
3. Doubtful uncertain whether sticker has been replaced.
9. Un-determined.

BAG-NUMBER: After entering sample data on the form, write down the given bag-number.

Invalid Samples



1) Insufficient Quantity
2) Appears Scratched or abraded
3) Not dried prior to mailing
4) Supersaturated
5) Diluted, discolored, or contaminated
6) Exhibits serum rings
7) Clotted or layered
8) No blood

10.7 Annexes to Chapter 7

10.7.1 Search strategy

The following search parameters were used:

Databases Pubmed

Date 1995 to present

Language English

Publication type Peer reviewed journals

Search terms

Searches will be in combination with the following:

1. PCR, polymerase chain reaction
2. Blood OR serum OR plasma
3. 1 of the pathogens

Leptospirosis

Leptospirosis, *leptospira*, Weil's disease, Weil's syndrome, canicola fever, canefield fever, nanukayami fever, 7-day fever, Rat Catcher's Yellows, Fort Bragg fever, Pretibial fever

Dengue

Dengue, DENV, flavivirus, flaviviridae

Japanese encephalitis

Japanese encephalitis, West Nile virus, WNV, JE, flavivirus, flaviviridae

Rickettsia

Rickettsia, tsutsugamushi, *Rickettsia typhi*, murine typhus, scrub typhus,

Bartonella

Bartonella, carrion, bartonella bacilliformis, oroya

Chikungunya

Chikungunya, chickungunya, chik, flavivirus

No searches were conducted for malaria as various experts at LSHTM were able to provide established PCR methods and protocols.

Inclusion criteria

The following inclusion criteria were used:

- PCR based methodology
- Primers and probe sequences available (not patented)
- Human clinical blood, serum or plasma samples are used (not culture extractions)
- Sensitivity and specificity determined with a reference method

Exclusion criteria

The following exclusion criteria were used:

- Studies that rely on sequencing or microarray
- "case studies" or "case reports"
- Animal focused studies
- Not using clinical samples
- Studies that do not determine PCR performance

Data extraction

Table 10.1: List of data extracted from the literature study

General information
Author
Article title
Country of origin

Study Characteristics
Aim/ objectives of the study
Study inclusion and exclusion criteria
Blinding of technicians

Participant/ Sample Characteristics
Participants:
Age
Sex
Fever length and status
Additional characteristics (HIV status, co-infections)
Number of samples
country
How were the samples acquired?
Sample type
Sample storage
Method of sample preparation

Technology

Sample volume processed
Nucleic Acid Extraction method
PCR:
multiplex
Platform
PCR method (SYBR green, Taqman, etc)
Primers and probe are given
Cycle conditions
Detection method (agarose gel, probes, melting curve,etc)
Subspecies differentiation
Reference method

Pathogen
Pathogenic subtypes detected
Target region

Outcome (qualitative)
Artificial specimen (plasmids, pos. controls, etc)
Sensitivity
Specificity
LDL
Clinical specimen
Sensitivity
Specificity
LDL
Correlation with reference method

Personal judgement on methodology/performance
Comments

10.7.2 PCR ASSAY SELECTION

Malaria

There were no searches conducted for malaria due to the vast experience with malaria PCR at the school. We therefore decided it was not useful to conduct searches but use the advised primers by malaria experts of LSHTM. The suggested assay showed good performance with DBS samples and showed excellent sensitivity(441).

Dengue

The Dengue primers target a non-coding region. Both ends of the open reading frame are flanked by an un-translated region (UTR). The genome organization is 5_-UTR-C-prM(M)-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3_-UTR, and this order is the same for other flavivirus (395). All assays target the 3' UTR region and are shown below:

Table 10.2: Table 10.2 Overview of dengue assays

Amplification region	Assay type	Assay type	Developer	copied method
3´ UTR, Non coding region	monoplex	SYBR green	Chutinimitkul(377), Shu(494)	
			bronzoni	Dos Santos(369)
		Taqman	Conceicao(495), Wu(496), gurukumar(372)	
	multiplex	Taqman	Ito(387), Chien(376), Kong(395), Irie	Munoz Jordan(385), warrilow(386)
			Callahan(375)	Naze(368)
			Drosten(381)	Poersch(393)
		Nested	Yamada(497)	Ito(387)
			Lanciotti	Saxena(391), matheus(95), Prado(96), Gomes(382), Klungthon(370), Kumaria(389), Raengaskulrach (498)
			Seah(499)	Singh(388)
		SYBR green	Yong(500)	
	X* mono/ duplo/ 4plex	taqman	Sadon(390), Bai(371), Johnson(392)	
			Harris	Kumaria(389)
			Wang(396)	

Although the Lanciotti primers are often used by others, the methodology is a nested PCR that amplifies a large region (>500bp). It is therefore not useful to use for our surveillance methodology. It is extremely difficult to make a 100% evidence based choice at this stage, but we have assessed three PCR methods using the following primers:

Table 10.3: Primer and probe sequences per selected assays

Paper	Target	Forward	Reverse	Probe
Gurukumar	3'UTR	GARAGACCAGAGATC CTGCTGTCT	ACCATTCCATTTTCTG GCGTT	AGCATCATTCCA GGCAC
Drosten	3'NCR	GGATAGACCAGAGATC CTGCTGT	CATTC CATTTTCTGG CGTTC plus CAATCC ATCTTGCG GCGCTC	CAGCATCATTCC AGGCACAG
Chien	Ns3	TTGAGTAAACYRTGC TGCCTGTAGCTC	GGGTCTCCTCTAAC C TCTAGTCCT	

The BLAST results indicate that none of the primers provide a perfect match, although BLAST shows only a selection of the matches. We therefore aligned all three PCR's to see how they match a selection of strains.

Alignment

The Chien reverse primer showed a few mismatches on the aligned sequences. The reverse primer of Drosten could not be aligned since it attaches at the end of the sequences. Since the last few basepairs (bp) of sequences are not really trustworthy, we cannot say how this primer would behave. The Gurukumar primers and probes had a good alignment on the strains and showed no mismatches. The alignment with strain 3 could not be used to test the performance of the reverse primer with the current alignments that were used. A new alignment to see if it works for strain 3 is required. The amplified product with Gurukumar primers is 68bp. While the Chien primers gave 121 bp product. The special MGB probe of Gurukumar seems to fit the DNA sequence of dengue well.

Table 10.4: Primer-dimer details of dengue assays

article	primer	Self dimer F	Self dimer R	Heterodimer
Gurukumar	F:58.8C, R:55.4°C	-4.62 kcal/ mole	-5.02 kcal/ mole	-8.2 kcal/mole F+R -6.62 kcal/mole R+P -6.69 kcal/mole F+P
Chien	F:60.4C, R:58.5°C	-6.34 kcal/ mole	-4.41 kcal/ mole	-4.41 kcal/mole

The primers of Gurukumar seemed to be one of the better options out of the various primer sets analysed. It appears that the primers are more likely

to heterodimer than the Chien primers. At this moment it is not possible to determine the effects of primer-dimer in the PCR reaction since it also greatly depends on the magnesium levels, PCR mix, and cycle conditions. The primers developed by Gurukumar showed 100% specificity when tested on 10 samples with various infections (malaria, JEV, West nile virus, rickettsia) according to the author. This specificity determination can be seen as a preliminary evaluation. The Chien primers were validated with yellow fever, JE, St. Louis encephaplitis, west Nile viruses and were optimised by using 109 Dengue strains.

Kong primers where also chosen because the WHO Dengue workgroup laboratories uses these primers relatively often.

Rickettsia

Since *O.tsutsugamushi* was initially in genus Rickettsia, Orientia and Rickettsia terminology are both used. We therefore combined the search for *Rickettsia typhi* and *Orienta tsutsugamushi*. Most assays targeted the same DNA region but varied on a more specific level as given in the table below.

Table 10.5: Overview of Rickettsia assays

Amplification region	Assay type	Developer	copied method	target
GltA	Sybr green	Roux(432)	Paris(401), Sousa(407)	Rickettsia spp
16s-RNA	Taqman	Sonthayanon (404)		tsutsugamushi
OmpB	standard Sybr green Mol. beacon	Roux(432) Blair(501), Parola Henry(362)	Souse(407)	
17-kDa	Nested	Leitner(403)		Rickettsia spp
	Taqman	Jiang(363)	Henry(362)	Rickettsia spp
47-kDa	Taqman	Jiang(363)	Paris(401), Paris (399)	tsutsugamushi
56-kDa	Taqman	Kramme(405)		tsutsugamushi
	Nested	Horinouchi (502)	Paris(399)	tsutsugamushi
	Nested	Furuya	Kim(503), saisonkorh (402)	tsutsugamushi
	SYBR green	Bakshi(406)		tsutsugamushi
GroEl	SYBR green	Paris(399)		tsutsugamushi

The assays reviewed were based on different assay types as SYBR green and Taqman. The different approaches to develop a suitable PCR assay make comparison more complicated. Based on the literature review, we selected some assays for further BLAST analysis. When looking at *O. tsutsugamushi*, the Kramme primers were highly successful in amplifying the target. It is however, unclear why the second probe has been developed. According to the author, it would be required to detect all the various strains but the second probe is less well suited than the first probe.

The disadvantage of the Jiang primer is that they are degenerative, a specific type of real time PCR assay. This type of assay has special features in how the PCR results should be interpreted with borderline cases.

Table 10.6: Primer-dimer details of Rickettsia assays

Article	Primer	Self dimer F	Self dimer R	Heterodimer
Jiang	F:56, R:53.3 °C	-17.98 kcal/mole	-8.16 kcal/mole	F&R;-6.91,
Kramme	F:50.8, R:52.2 °C	-11.42 kcal/mole	-3.91 kcal/mole	F&R:-4.95, R&P;-2.94, F&p;-5.85

Theoretically, the high self dimer effect of both forward primers is unexpected. The specificity of the primers developed by Jiang has been validated with 26 *O.tsutsugamushi* strains, 17 Rickettsia strains and 18 other bacterial DNA samples. No further details are given considering which pathogens have been analysed for the Kramme assay, other than "an extensive panel has been used".

The Oxford collaborating hospital has done extensive work in developing PCRs for Rickettsia spp. Since they participate in this project, we will include the assay developed by D. Paris MD as well.

Leptospira

For *leptospira*, the few articles that were selected made a comparison on PCR primer level relatively easy. There were 4 amplification regions used as can be seen in the table below. Most authors developed an assay themselves.

Table 10.7: Overview of Leptospira assays

Amplification region	Assay type	Developer	Copied method
LipL32	LUX	Roczek(442)	
	SYBR green	Levett(414)	
	Taqman	Stoddard(408)	
16s/23sRNA	Nested PCR	Djadidad(409), Kositanont(411)	
	Taqman	Smythe(364)	Slack(413)
SecY	SYBR green	Ahmed(415)	
pLIPs60	2x mono	Gravekamp(504)	Fonseca(412, 416), Ooteman(410)

The pLIPs60 has been found to provide less efficient PCR's and less reproducible than other primers analyzed in this review. Based on the data extracted and preference of methodology, we decided to focus on three methods:

Table 10.8: Details of Leptospira assays

Paper	Target	Forward	Reverse	Probe
smythe	16srDNA	cccgcgtccgatt ag	tccattgtggccgR$^{a/g}$acac	ctcaccaaggcgacga tcggtagc
Roczek	Lip32	TAATCG CTGAAA TGGG	AGCAGACCAACAGAT GCAACG	
Ahmed	secY	GCGATTCAGT TT AATCCTGC	GAGTTAGAGCTCAAAT CTA	Sybr green

The three assays were used for BLAST analysis. All three assays performed good according to BLAST searches. Based on these results, alignments were made with available sequences online. Because the Smythe probe detects other bacteria according to BLAST searches, we have decided not to include these primers for further analysis. It would be highly unlikely that Zebrafish would become an issue when using the Ahmed primers. Additionally, the Ahmed primers are developed by Royal Tropical Institute (KIT) Amsterdam. Since we will use the samples provided by KIT we will include their primers in the analysis.

Alignment results

The Rockzek primers attach well on the various *Leptospira* species and strains in our alignment evaluation. The amplified length is long; 247bp. The melt temperature of Rockzek primers are relatively high (70.5°C) but since melt curve and cycle detection can be used with a assay type called Light-upon-

extensions (LUX), it should not cause any problem. LipL32 nucleotide BLAST resulted in only *Leptospira* related bacterial results. Intraspecific attachment was not tested. The amplified length is 204bp. The melt temperature is 72°C, which is even higher than for Roczek.

Table 10.9: Primer-dimer details of selected Leptospira assays

Article	Primer	Self dimer F	Self dimer R	Heterodimer
Roczek	F:54.8C, R:58.1°C	-3.61kcal.mole	-7.05 kcal/mole	-5.02 kcal/mole
Ahmed	F:51.8C,R:47.8°C	-4.99 kcal/mole	-9.49 kcal/mole	-4.99 kcal/mole

It seems that both assays are quite good and could be used for our assay. The final choice depends on the methodological preference. The Roczek primers have not been analysed for specificity, other than BLAST results. The specificity of Ahmed primers has been validated with over 46 different pathogens by the authors and showed good results. Both primer sets have been ordered and tried in the laboratory.

Chikungunya

For chikungunya, the various PCR assays were analysed and categorised. All assays targeted the same DNA region E1.

Table 10.10: Overview of PCR assays reviewed

Amplification region	Assay type	Developer	Copied method
E1	SYBR green	Santhosh(424)	
		Ho(419)	
	Taqman	Panning(421)	Panning(420, 422),
		Laurent(425)	Staikowsky(358), Naze (368)
		Edwards (418)	
	standard	Dash (379)	
		Hasebe (505)	Rohani (506)
	LAMP	Parida (426, 507)	

Most primers developed are using plasma/serum. Surprisingly, one group has developed 3 different PCR's (SYBR green, Taqman and LAMP assay) with different primers. Basically, all articles are from groups based in India, France or Germany.

All primers target the same region which allows primers to be easily analysed by alignment. First a selected group of primers were selected based on performance and quality of research performed.

Table 10.11: Sequence data of selected assays

Paper	Target	Forward	Reverse	Probe
Panning 1	E1	TGATCCCGACTC AACCATCCT	GGCAAACGCAG TGGTACTTCCT	TCCGACATCATC CTCCTTGCTGGC
Panning 2	IOS	CCGACTCAACC ATCCTGGAT	CGGCAGACGCA GTGGTACTTCCT	TCCGACATCATC CTCCTTGCTGGC
Ho	E1	ggcagtggtcccag ataattcaag	gctgtct agatccac cccata catg	
Laurent	E1	AAGCTCCGCGTC CTTTACCAAG	CCAAATTGTCC TGGTCTTCCT	CCAATGTCTTCAG CCTGGACACCTTT
Dash	E1	ACGCAGTTGA GCGAAGCAC	CTGAAGACATTG GCCCCAC	

All assays selected above were analysed with BLAST which gave a perfect match for all primers and probes reviewed. While the Panning primers scored very well, Dash primers seemed to work just as well. Dash primers have the huge advantages that they can be combined with general dengue primers. According to Ho *et al.* the primers have a few weaknesses regarding strains in Africa. Laurent primers gave good results as well but needed adjustment since this assay is a molecular beacon type which works as Taqman assay but is less suitable to optimize. The Laurent primers are used by the collaborating partners of our Laos collaborators and gave good results. The Laurent primers were adjusted to Taqman format and tried in the laboratory.

Bartonella bacilliformis

For *Bartonella bacilliformis*, the assays targeted 5 different regions which made comparisons more complicated. Additionally, the concern that primers could bind and amplify other regions appeared to be a true issue.

Table 10.12: Overview of reviewed assays

Amplification region	Assay type	Developer	Copied method
ribC	standard	Bereswill (435)	
16s-23sRNA	standard	Jensen (434)Roux (432) Maggi (431)	
		Garcia-esteban (360) Norman (508) (excluded)	zeaiter (433)
		Birtles (509) Houpikian (510)	Patented!
groEl	Taqman	Callison (429)	
ftsZ	standard	zeaiter (430)	
16s	standard	Relman (511)	Koehler (512)

The primers developed by these authors all amplify a larger region than would be preferred when using Taqman. The aim of these primers is to amplify all Bartonella species and to allow differentiation, except the groEL primers. The groEL primers are specifically developed for *B.bacilliformis*. Maggi et al showed that the primers developed by Jensen, Houpikain, Birtles and Roux all amplify the bacteria mesorhizibium species, which appears to be relatively common in water.

Table 10.13: Sequences of selected PCR assays

Paper	Target	Forward	Reverse	Probe
Maggi	ITS	AGATGATGATCCCAA GCCTTCTGG	TGTTCTYACAACAAT GATGATG	
Callison	groEL	CTTA AGCG CGGAATTGATGC	CTGAGGTTTGGATCT TCTTCGC	CTGTTGAAGCGGTTG TTGCAGATCTTTTC

When comparing the two assays with BLAST, differences in primer binding capabilities are reviewed. The interpretation of Callison's primers is difficult.

It seems that the primers primarily amplify the bacilliformis species and not any other *Bartonella bacilliformis* strains. Depending on the BLAST database, it could be that there are not many groEL sequences widely available as 16s-23sRNA region. The primer and probe seems to weakly bond to other pathogens and even human DNA but this could be too weak to cause any problem.

The Maggi primers seem to work very well to identify many Bartonella species. This method is intended to be performed with agarose gel for the differentiation and identification of the Bartonella species. It has been commented that the adjusted primers developed by Maggi do not allow a good differentiation of *B.henselae* since the amplicon size only differs 6-8bp with some other strains. For our project, this is not a major problem. The amplified product is also very long for the Maggi primers, which amplifies over 450bp. This amplicon size is ideal for gel based detection but is not preferable with DNA isolated from DBS since the chance of fragmentation is larger than using serum.

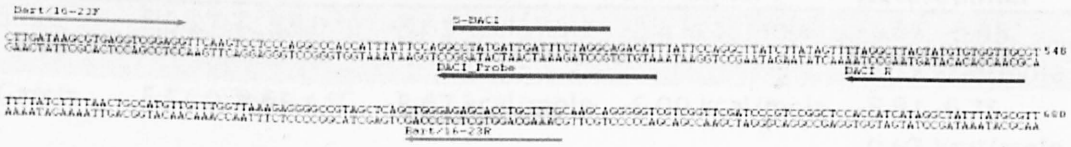
Table 10.14: Primer-dimer details of selected Bartonella PCR assays

Article	Primer	Self dimer F	Self dimer R	Heterodimer
Maggi	F:58,7,R: 50.1 °C	-5.02 kcal/mole	-5.24 kcal/mole	-3.36 kcal/mole
Callison	F:54.9, R:55.8 °C	-10.36 kcal/mole	-4.62 kcal/mole	-6,75,-6,75,-6,75 kcal/mole

Both primer sets have acceptable self and heterodimer features. Both primer assays have not been thoroughly validated for the specificity. The Maggi primers are adjusted existing primers that have been tested for other bacteria. There is little known about the Callison primers.

Development of *Bartonella bacilliformis* PCR

After the extensive literature study was performed for *Bartonella bacilliformis*,



no valuable Real-Time PCR assay could be found. An assay developed for reverse line blotting showed heterogenous Bartonella primers suitable for all Bartonella species. Based on these and the specific probe for *Bartonella bacilliformis*, we optimised and converted this PCR based reverse line blotting assay into a Taqman based Real Time PCR assay(360). The figure below shows the place of the initial primers (Bart/16-23F and Bart/16-23R), the developed probe (red) and the new reverse primer BACI R.

Figure 10.1: Shows the DNA sequence of Bartonella bacilliformis and the location of Garcia-Esteban primers (Bart/16-23F, Bart/16-23R and S-BACI) and new primers and probes (BACI R and BACI-probe)

These primers were chosen based on BLAST searches and alignment of 8 bacilliformis strains. After the primers and probe were ordered and used successfully, we found another strain of *B.bacilliformis* that had 1 mismatch at two places with the reverse primer. To see what the effect would be, we ordered 3 more plasmids containing 1 of the two possible mismatch places and one containing both mismatches. Although it appeared that the PCR efficiency was slightly negatively influenced, the effect was too little to consider redeveloping the reverse primer. Below are given the primer details.

Table 10.15: PCR sequences of selected Bartonella assays

Paper	Target	Forward	Reverse	Probe
Smit	16-23S	TTGATAAGCGTGA GGTCGGAGG	GCAACCACACATAGT AAGCCTAA	ATgTCTgCCTAgAAAT CAATCATAggCC
Garcia	16-23s	GCCYCCTTGCGGTTA GCACAGCA	CCTTCAGTTMGGCTG GATC	CTCGCCCTTAGTTGC CAGCATT

Table 10.16: Primer- dimer details of selected Bartonella assays

Article	Primer	Self dimer f	Self dimer r	Heterodimer
Smit	F:58.7, R: 55.0 °C	-3.61 kcal/mole	-3.4 kcal/mole	-4.61, -6.95, -8.77 kcal/mole
Garcia	F:54.0, R:65.4 °C	-5.67 kcal/mole	-5.09 kcal/mole	-8.81,-6.75, -9.67 kcal/mole

The newly developed assay shows good primer qualities, the primer melting temperatures are closer together and self or heterodimer binding capabilities have a lower kcal/mole values, indicating that primer-dimers are less likely than with the Garcia assay.

10.7.3 Plasmid Sequences

Plasmids that were developed and ordered for each of the assays are given below.

Gene name: Bart bacilliformis, Length: 221 bp, Vector name: pUC57,
Quality grade: Research Grade (Predominantly supercoiled),
Sequence:

GCGTGAGGTCGGAGGTTCAAGTCCTCCCAGGCCCACCATTTATCCAGGCCT ATGATT-
GATTTCTAGGCAGACATTTATTCCAGGCTTATCTTATAGTTTTAGGCTTACTATGTGTG-
GTTGCGTTTTTATCTTTTAACTGCCATGTTGTTTGGTTAAAGAGGGGCCGTAGCTCAG
CTGGGAGAGCACCTGCTTTGCAAGCAGGG

Gene name: Leptospira interrogans, Length: 215 bp, Vector name: pUC57,
Quality grade: Research Grade (Predominantly supercoiled),
Sequence:

CAGCGATTCAGTTTAATCCTGCAGAATTGGCTGAGAATTTGAAAAAATACGGTGG
GTTCATTCCAGGAATTCGTCCGGGTTCTCACACAAAAGAATACATTGAAAAAGTGTTA
AATAGAATCACTCTTCCCGGAGCTATGTTTCTTGCAGGTTTGGCATTAGCACCTTATAT
TATTATAAAATTCTTAGATTTGAGCTCTAACTCCGGCGGTGGA

Gene name: Plasmodium vivax, Length: 211 bp, Vector name: pUC57,
Quality grade: Research Grade (Predominantly supercoiled),
Sequence:

CCGATAACGAACGAGATCTTAACCTGCTAATTAGCGGCAAATACGATATATTCTTACGT
GGGACTGAATTCGGTTGATTTGCTTACTTTGAAGAAAATATTGGGAAACGTAACAGTT
TCCCTTTCCCTTTTCTACTTAGTTCGCTTTTCATACTGTTTCTTTTTCGCGTAAGAATGTA
TTTGCTTGATGTAAAGCTTCTTAGAGGAACGAT

Gene name: Plasmodium falciparum, Length: 260 bp, Vector name: pUC57,
Quality grade: Research Grade (Predominantly supercoiled),
Sequence:
GTCTGGTTAATTCCGATAACGAACGAGATCTTAACCTGCTAATTAGCGGTAAGTACA
CTATATTTTTATTTGAAATTGAATATAGGTAATTATACATGTTTATTCAGTGTTCAAATTA
GGATATTTTTTTATTAAAATATTCTTTTCCCTGTTCTACTATAATAATTTGTTTTTTTTTAC
TCTATTTCTCTCTTCTTTTAAGAATGTACTTGTTTGATTAAATAAAGCTTCTTAGAGGAA
CAGTGTGTATCTAACACAAG
Gene name: Plasmodium ovale, Length: 234 bp, Vector name: pUC57,
Quality grade: Research Grade (Predominantly supercoiled),
Sequence:
CTGGTTAATTCCGATAACGAACGAGATCTTAACCTGCTAATTAGCGGCGAATACGTTAT
ATTCCTACTTGAAATTGAATATAGCTGAATTTGCTTATTTTGAAGAATATATTAGGATGC
ATTATAGTGTCCTTTTCCCTTTTCTACTTA ATTCGCTATTCATGCTGTTTCTTTTTTGTGTA
GAATGTATTCGTTTGATTGTAAAGCTTCTTAGAGGAACGATGTGTGTCTAAC

Gene name: Plasmodium malariae, Length: 249 bp, Vector name: pUC57,
Quality grade: Research Grade (Predominantly supercoiled),
Sequence:
CTGGTTAATTCCGATAACGAACGAGATCTTAACCTGCTAATTAGCGGTAAATACACTAT
ATTCTTAAGTGAAATTAGAATATAGATAAATTGTGCTAATTTTGATTAAAATATTAGAATG
TTTTTTTTAATAAAAACGTTCTTTTCCCTTTTTTTCTTAATTATGCATATTTATTCTTTTTCT
TTTTTCGCATAAGAATGTATTTGCTTAATTGTAAAGCTTCTTAGAGGAACGATGTGTGTC
TAACAC

Gene name: *Orienta tsutsugamushi*, Length: 189 bp, Vector name: pUC57,
Quality grade: Research Grade (Predominantly supercoiled),
Sequence:

ACAGTACTTTGCAACGAATCGTGAAAAGATGATTACTGAATTTGAAAATCCTTATATTTT
GCTATTGGATCAGAAGGTATCTACAGTGCAGCCACTGGTTCCTGTGCTTGAAGCTG
TTGCTCACACTGGCAAGCCATTAGTATTGATTGCTGATGATGTAGACGGAGAAGCTCT
TACTGCATTGATATT

Gene name: Rickettsia typhi, Length: 167 bp, Vector name: pUC57,
Quality grade: Research Grade (Predominantly supercoiled),
Sequence:

AAGTCTTACCATAACAGGTCATGGTATTACTGCTCAACAAGCTGCTACTACAAAAAG
TGCTCAAAATGTTGTTTCAAAAGTTAATGCTGGTGCTGCTATTAACGATAATGATCTTA
GCGGTGTAGGATCAATAGACTTTACTGCTGCGCCTTCTGTATTAGAATTTA

Gene name: Bartonella Bacilliformis mismatch combi G-T537, T552 deletion, Length: 220 bp, Vector name: pUC57,
Quality grade: Research Grade (Predominantly supercoiled),
Sequence:

GAGCGCGCGCTTGATAAGCGTGAGGTCGGAGGTTCAAGTCCTCCCAGGCCCACCA
TTTATTCCAGGCCTATGATTGATTTCTAGGCAGACATTTATTCCAGGCTTATCTTATAGT
TTTAGGTTTACTATGTGTGGTGCGTTTTTATCTTTTAACTGCCATGTTGTTTGGTTAAAG
AGGGGCCGTAGCTCAGCTGGGAGAGCACCTGCTTTGCAAGCAGGG

Bartonella Bacilliformis mismatch combi G-T537, T552 deletion-Mutagenesis:
Variant name: Bartonella Bacilliformis mismatch 1 T552 deletion, Vector: pUC57,
Quality grade: Research Grade (Predominantly supercoiled),
Variant sequence:

GAGCGCGCGCTTGATAAGCGTGAGGTCGGAGGTTCAAGTCCTCCCAGGCCCACCA
TTTATTCCAGGCCTATGATTGATTTCTAGGCAGACATTTATTCCAGGCTTATCTTATAGT
TTTAGGCTTACTATGTGTGGTGCGTTTTTATCTTTTAACTGCCATGTTGTTTGGTTAAA
GAGGGGCCGTAGCTCAGCTGGGAGA GCACCTGCTTTGCAAGCAGGG

Bartonella Bacilliformis mismatch combi G-T537, T552 deletion-
Mutagenesis:
Variant name: Bartonella Bacilliformis mimatch2 G-T537,
Quality grade: Research Grade (Predominantly supercoiled),
Variant sequence:

GAGCGCGCGCTTGATAAGCGTGAGGTCGGAGGTTCAAGTCCTCCCAGGCCCACCA
TTTATTCCAGGCCTATGATTGATTTCTAGGCAGACATTTATTCCAGGCTTATCTTATAGTT
TTAGGTTTACTATGTGTGGTTGCGTTTTTATCTTTTAACTGCCATGTTGTTTGGTTAAAG
AGGGGCCGTAGCTCAGCTGGGAGA GCACCTGCTTTGCAAGCAGGG

Gene name: dengue 1 Lp- Goffart, Length: 135 bp, Vector name: pUC57,
Quality grade: Research Grade (Predominantly supercoiled),
Sequence:

AGGACTAGAGGTTAGAGGAGACCCCCCGCATAACAATAAACAGCATATTGACGCTG
GGAGAGACCAGAGATCCTGCTGTCTCTACAGCATCATTCCAGGCACAGAACGCCAG
AAAATGGAATGGTGCAGTTGAAT

Gene name: dengue 2 Lp- Goffart, Length: 138 bp, Vector name: pUC57,
Quality grade: Research Grade (Predominantly supercoiled),
Sequence:

GTCTCACTGGAAGGACTAGAGGTTAGAGGAGACCCCCCCAAAACAAAAAACAGCA
TATTGACGCTGGGAAAGACCAGAGATCCTGCTGTCTCCTCAGCATCATTCCAGGCAC
AGAACGCCAGAAAATGGAATGGTGCT

Gene name: dengue 3 Lp- Goffart, Length: 127 bp, Vector name: pUC57,
Quality grade: Research Grade (Predominantly supercoiled),
Sequence:

CCTCCTTGCAAAGGACTAGAGGTTAGAGGAGACCCCCCGCAAATAAAAACAGCATA
TTGACGCTGGGAGAGACCAGAGATCCTGCTGTCTCCTCAGCATCATTCCAGGCACA
G AACGCCAGAAAATG

Gene name: dengue 4 Lp- Goffart, Length: 127 bp, Vector name: pUC57,
Cloning strategy: pUC57,
Quality grade: Research Grade (Predominantly supercoiled),
Sequence:

CTTCTGGTGGAAGGACTAGAGGTTAGAGGAGACCCCCCCAACACAAAAACAGCATA
TTGACGCTGGGAAAGACCAGAGATCCTGCTGTCTCTGCAACATCAATCCAGGCACA
GAGCGCCGCGAGATG

Gene name: Leptospira Meyeri, Length: 233 bp, Vector name: pUC57,
Quality grade: Research Grade (Predominantly supercoiled),
Sequence:

ACTTTTATACAGCGATTCAGTTTAATCCTGCAGAATTGGCTGAGAATTTGAAAAAATAC
GGTGGATTCATTCCAGGAATTCGTCCGGGTTCTCACACAAAAGAATACATTGAAAAA
GTGTTAAATAGAATCACTCTTCCCGGAGCTATGTTTCTTGCAGGTTTGGCATTAGCAC
CTTATATTATTATAAAATTCTTAGATTTGAGCTCTAACTCCGGCGGTGGATCTTTGGTT

Gene name: Leptospira santorosai, Length: 233 bp, Vector name: pUC57,
Quality grade: Research Grade (Predominantly supercoiled),
Sequence:

ACTTTTACACTGCGATTCAGTTCAACCCTGCGGAGTTGTCCGAAAATCTGAAGAAATA
CGGCGGGTTCATTCCAGGCATTCGTCCCGGTTCTCACACAAAAGAATACATCGAAAA
GGTGTTAAACAGAATCACACTTCCCGGCGCGATGTTCCTCGCGGGATTGGCTCTGG
CTCCTTACATCATCATCAAATTCTTAGATTTGAGCTCCAACTCCGGAGGCGGATCTTTG
GTT

Gene name: Leptospira Weilli, Length: 225 bp, Vector name: pUC57,
Quality grade: Research Grade (Predominantly supercoiled),
Sequence:

ACTTTTATACCGCGATTCAGTTTAACCCTTCGGAGTTGGCTGAAAATTTGAAGAAATAC
GGCGGGTTCATTCCGGGAATTCGTCCAGGTTCTCACACGAAAGAATACATCGAAAAA
GTGTTAAACAGAATCACTCTTCCGGGTGCGATGTTTCTCGCTGGATTGGCATTAGCA
CCTTATATTATTATAAAATTCTTAGATTTGAGCTCCAACTCCGGCGGCGGAT

Gene name: Leptospira Kirschneri, Length: 225 bp, Vector name: pUC57,
Quality grade: Research Grade (Predominantly supercoiled),
Sequence:

ATTTTTATACTGCGATTCAGTTTAACCCTGCGGAGTTGGCTGAGAATCTGAAAAAATA
CGGCGGATTTATCCCAGGAATTCGCCCCGGTTCTCATACAAAAGAATACATCGAAAA
AGTGTTAAATAGAATCACCCTTCCTGGAGCCATGTTTCTTGCCGGTTTGGCATTAGCA
CCTTATATTATTATAAAATTCTTAGATTTGAGCTCCAACTCCGGAGGTGGGT

Gene name: Chikungunya, Length: 230 bp, Vector name: pUC57,
Quality grade: Research Grade (Predominantly supercoiled),
Sequence:

CATCTGCATCAGCTAAGCTCCGCGTCCTTTACCAAGGAAATAACATCACTGTAACTGC
CTATGCAAACGGCGACCATGCCGTCACAGTTAAGGACGCCAAATTCATTGTGGGGC
CAATGTCTTCAGCCTGGACACCTTTCGACAACAAAATTGTGGTGTACAAAGGTGAC
GTCTATAACATGGACTACCCGCCCTTTGGCGCAGGAAGACCAGGACAATTTGGCGA
TATC